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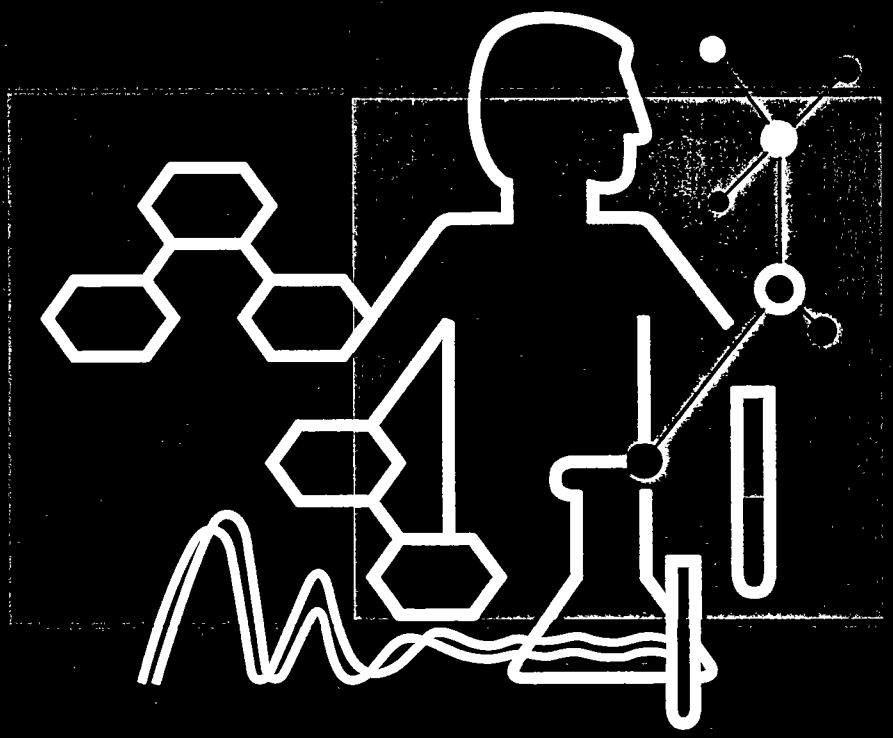
Training Module No. 4

GENERAL SCIENTIFIC PRINCIPLES OF CHEMICAL SAFETY



UNEP

IOMC

WORLD HEALTH ORGANIZATION

IPCS

Training Module No. 4

GENERAL SCIENTIFIC PRINCIPLES OF CHEMICAL SAFETY

This training module was scientifically edited by Dr L. Fishbein, Fairfax, Virginia, USA.

Produced under the joint sponsorship of the United Nations Environment Programme, the International Labour Organization and the World Health Organization, and within the framework of the Inter-Organization Programme for the Sound Management of Chemicals

The International Programme on Chemical Safety (IPCS), established in 1980, is a joint venture of the United Nations Environment Programme (UNEP), the International Labour Organization (ILO), and the World Health Organization (WHO). The overall objectives of the IPCS are to establish the scientific basis for assessing the risk to human health and the environment from exposure to chemicals, through international peer-review processes, as a prerequisite for the promotion of chemical safety, and to provide technical assistance in strengthening national capacities for the sound management of chemicals.

The Inter-Organization Programme for the Sound Management of Chemicals (IOMC) was established in 1995 by UNEP, ILO, the Food and Agriculture Organization of the United Nations, WHO, the United Nations Industrial Development Organization, the United Nations Institute for Training and Research, and the Organisation for Economic Co-operation and Development (Participating Organizations), following recommendations made by the 1992 United Nations Conference on Environment and Development to strengthen cooperation and increase coordination in the field of chemical safety. The purpose of the IOMC is to promote coordination of the policies and activities pursued by the Participating Organizations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

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GENERAL SCIENTIFIC PRINCIPLES OF CHEMICAL SAFETY

FOREWORD

The principal focus of this work is to further the objectives of the IPCS in its elaboration and evaluation of the effects of chemicals on human health and the environment and the development of methodology, testing methods and risk evaluation by highlighting the basic tenets of the cornerstone, toxicology. Toxicology is increasingly recognized to be a dynamic discipline where change is being driven by both technological and conceptual advances. Hence this report will reflect the panoply of interactions between chemicals and living organisms via the broad spectrum of animal studies which measure the toxic effects resulting from both short-term and long-term exposures at low and high concentrations of agents, interspecies extrapolations, mechanism of bioactivation and detoxification as well as toxicokinetics and toxicodynamics.

The essentials of toxicology are extended into risk assessment (and its limitations and uncertainties) including hazard identification, dose response, exposure assessment, risk characterization and risk management. Risk assessment is recognized to be not one procedure with one outcome, but a complex of related activities, all of which inform an overall judgement as to health risk. Future developments in the many areas of testing and harmonization of cancer and non-cancer risk assessments are also featured.

This training module builds on and updates the original text (post Task Group draft, April 1995) of the IPCS document (originally intended for publication in the Environmental Health Criteria series) entitled General Principles and Methods for Chemical Safety (Human Health Protection). In place of the many original textual references, a selected bibliography has been added.

It is hoped that this work will serve as a useful guide and timely review for a broad audience including disciplines of general, industrial, environmental and experimental toxicologists, chemists, occupational health physicians and those involved in risk assessment, risk management and risk communication on various local, national and international levels.

1. CHEMICAL SAFETY FOR HUMAN HEALTH PROTECTION: CONCEPTS AND APPLICATION

1.1 Definition of chemical safety

The International Programme on Chemical Safety (UNEP-ILO-WHO) defines chemical safety as "the prevention and management of the adverse effects, both short- and long-term, to humans and the environment from the production, storage, transportation, use and disposal of chemicals".

Chemical safety (as defined by IUPAC in 1993) involves consideration of hazard and risk:

Hazard: Set of inherent properties of a substance, mixture of substances or a process involving substances that, under production, usage or disposal conditions makes it capable of causing adverse effects to organisms or the environment, depending on the degree of exposure; in other words, it is a source of danger.

Risk: 1. Possibility that a harmful event (death, injury or loss) arising from exposure to a chemical or physical agent may occur under specific conditions; 2. Expected frequency of occurrence of a harmful event (death, injury or loss) arising from exposure to a chemical or physical agent under specific conditions.

1.2 The need for chemical safety for human health protection

A general statement that forms the core of chemical safety is that no chemical is entirely safe but every chemical can be used safely (although this may require a high degree of control in some cases). Like all generalizations this cannot cover every situation but it gives general direction to chemical safety and its related activities.

All humans are in contact, from conception to death, intentionally or unintentionally, with a multitude of chemicals, natural and man-made, in food and drinking-water, in the general environment, home, and workplace, and in ambient air. These chemicals may have local effects on the surfaces where they contact the body - skin, eyes, respiratory tract, gastrointestinal tract - and, if absorbed, may have systemic effects. There is exposure to chemicals absorbed from the environment, and to their metabolic transformation products, some less toxic due to detoxification processes but some as toxic or even more toxic than the parent chemical. Depending on the dose, a chemical may have no detectable adverse effects or a spectrum of effects ranging from minor local change and discomfort up to severe systemic effects culminating in death. Adverse effects may be acute, delayed, chronic, reversible or irreversible, or may affect progeny of exposed individuals.

The use of chemicals in practically every aspect of life has grown very rapidly over the last few decades. Out of over 11 million known chemicals, about 100 000 are being produced on industrial scale and about 1000–2000 new chemical entities are being introduced each year. Human beings come into contact with many of the chemicals in

commercial production, as well as thousands of substances of natural origin, which may be present as pollutants and contaminants in food, environmental media and commercial products. It is evident that a substantial use of chemicals is involved in meeting the social and economic goals of the world community, but many of these can pose substantial health risks due to their toxicity to the living organisms. It is therefore important that their hazards are recognized so that risk situations can be eliminated or otherwise controlled and adverse effects prevented.

Chemical exposure is not uniform, and, depending on location, populations may be exposed to different chemicals, exposure patterns, and magnitudes of exposure fluctuating with time. Toxic responses are influenced by individual factors, including metabolic function, which is largely genetically determined, and state of health. Consequently, the type and degree of risk can vary from one individual, group, or population to another. In the working environment, exposure to chemicals may be high but of limited duration and affect only small groups, whereas the general population, including vulnerable groups such as infants, pregnant women, the elderly, the chronically ill and the malnourished, can be exposed continuously to low concentrations of chemicals. However, in the current world situation, it cannot be assumed that all occupationally exposed groups are healthy adults: in some countries child labour is still used while adult workers can suffer from endemic chronic diseases and malnutrition.

In a general sense, the toxicity of a substance is its capacity to cause injury to a living organism. It is an inherent property of every chemical substance. The division of chemical into "toxic" and "nontoxic" or "harmful" and "safe" is not scientifically justified. Even apparently safe substances can damage organisms, when administered in large amounts. On the other hand, very toxic chemicals can cause no adverse effects when there is exposure to sufficiently small amounts. Thus, toxicity is a relative property of a chemical which is useful in comparing one chemical with another. Toxic potential should be always defined in relation to the quantity of a substance administered or absorbed (dose), the way in which the substance enters the organism (route of exposure) and its distribution in time (single dose or repeated doses), the type and severity of adverse effects, the time needed to produce effects, the nature of the organism(s) affected and other relevant conditions (IUPAC, 1993).

1.3 Historical development of chemical safety

The knowledge that chemicals are poisonous has been known for millennia. Exposure to highly toxic chemicals of natural origin, such as poisonous plants, animals, or biological contaminants of food (e.g. ergot) that produced either serious illness or death allowed the connection between exposure and effect to be made reliably. With increasing knowledge, the selective use of poisons for murder, suicide, execution, or political assassination developed. In the case of suicide, execution or assassination reliability was the desired quality, whereas for murder it was preferable that the poison had effects similar to a natural disease or sufficiently delayed to avoid suspicion falling on the poisoner. The fact that some compounds could have beneficial therapeutic effects at one dose and harmful or even fatal effects at a higher dose was also known for centuries. The basics of modern toxicology were formulated in the 16th century by the

Swiss physician, Paracelsus (Philippus Aureolus Theophrastus Bombastus von Hohenheim: 1493-1541) who believed in observation and experimentation and who recognized that toxic and pharmacological properties were related to both the substance and to the dose; in 1538 he stated "What is it that is not a poison? All things are poisons and none that are not. Only the dose decides that a thing is not poisonous." However, because he insisted in teaching and writing in Swiss German instead of Latin the impact of his ideas was restricted. It was not until 1814 that the first textbook of toxicology was written by the Spanish physician, Mattieu Orfila (1787–1853) working in Paris, who for the first time defined toxicology as the study of poisons, systematically examined available chemical and biological information on poisons, and emphasized the importance of chemical analysis in the investigation of poisoning.

In the case of the workplace, the knowledge that certain occupations and working environments were associated with specific diseases is also of long standing. The toxic risks of mining and smelting certain ores were well known in ancient times and the first occupational disease (lead colic) was described in about 400 BC in the slaves working in lead mines by Hippocrates. Galen (131–201), a Greek physician who practised in Rome, described a number of occupations and associated diseases including lead poisoning in lead smelter workers and the danger of acid mist in copper mines. [add: Vitruvius and extrapolation from lead poisoning of miners to poisoning of general population by lead from water pipes] Centuries later Paracelsus (1493–1541) and a contemporary, Georgius Agricola described occupational diseases of miners and metal workers. Paracelsus wrote a book on occupational diseases which identified diseases of miners, which he attributed to vapours from the metals and which he advised should be avoided. Bernardo Ramazzini (1633–1714), Professor of Medicine at the University of Padua is considered a father of industrial medicine, whose studies of occupational diseases and advocacy of protective measures for workers encouraged eventual passage of factory safety and workmen's compensation laws. His Diseases of Workers, was the first comprehensive work on occupational diseases, outlining the health hazards of irritating chemicals, dust, metals, and other abrasive agents encountered by workers in 52 occupations and was the only book on the subject available for the next 200 years.

Another landmark was the report in 1775 by Percivall Pott (1714–1781) of the prevalence of scrotal epitheliomata and carcinoma in young English chimney sweeps, attributable to their poor working conditions, having to climb inside chimneys to clean them manually with consequent gross contamination of clothing by soot, and lack of personal hygiene including wearing unchanged soot-contaminated trousers. This resulted in the Chimney Sweeps Act, passed in 1788, that can be considered as a prototype for chemical safety-related legislation.

Occupational safety and health legislation was first enacted in some countries in the late 1800s–early 1900s. Defined occupational exposure limits appeared in the 1930s. The term itself was introduced by the International Labour Conference in 1977. Specifically, it relates to the level of occupational exposure below which there should be no health hazard for the workers and no deterioration in the degree of comfort that is required to maintain production and to keep the risk of accidents to a minimum. The first lists of maximum allowable concentrations of airborne toxic substances at the

workplace were issued between 1933 and 1938 in the USSR, the USA and Germany. Exposure limits may vary from country to country, depending on the health criteria applied, the safety margin they incorporate, and the extent of their being influenced by the technical and socioeconomic factors. Exposure limits are constantly being revised, reflecting the current scientific evidence of the health impact and the health policy followed. The application of control to other types of chemicals, for example food, drinking-water, and air quality followed later in the 1940s.

1.4 Chemical safety concepts and application

The achievement of safety in any situation is an active and continuing process. The principles and methods of hazard and risk recognition, assessment, and appropriate management to eliminate, or at least ameliorate, risks are being applied in fields as diverse as safety at sea, in aviation flight safety, road traffic, and in many industries, as well as in chemical safety. Basic principles of chemical safety are the identification of hazard and the evaluation and management of risk. General principles of chemical safety include recognition of the existence of hazard and risk; risk analysis/risk assessment (risks are identified and probabilities of occurrence determined); risk evaluation (consideration of aspects such as preventive and precautionary measures, their technical feasibility, extent of protection (who and for how long), cost and risk/benefit, and management options); risk management (including voluntary and regulatory options, standard setting and enforcement); risk perception and communication; and, education and building of awareness at all levels ("accidents are risk situations waiting for people to make them happen").

Chemical safety is involved in all the stages of a chemical's existence, including its natural presence in the environment or as a synthetic chemical, through extraction or processing with the possible production of chemical waste, formulation, distribution and transportation, use, disposal, environmental release and fate, with emphasis on assessment of risk, dissemination of information including an important educational component, and practical application in managing risks.

All chemicals are not of equal concern. Their toxicity, potential health hazard, production volume and use pattern differ significantly. It is therefore practicable and advisable that priorities are established and followed for risk assessment research and risk management. The general criteria for giving priorities usually include:

- indications or suspicion of hazard to human health and type and severity of existing or potential health effects
- type and size of exposed population
- extent of production
- nature and extent of use(s)
- potential for accumulation in biota
- potential for persistence in the environment

If an adverse health effect(s) is likely to occur in the defined exposure conditions this exposure situation is considered a risk to health. The potential of a chemical to

induce adverse health effects is a matter of concern in safety consideration. The distinction between adverse and non-adverse effects is difficult and often involves scientific judgement. In a general sense, an adverse effect could be defined as reversible or irreversible effects on the organism which affect its health or well-being. These usually involves changes in morphology, physiology, growth, development or lifespan of an organism which results in impairment of functional capacity, or of the capacity to compensate for additional stress, or cause an increase in susceptibility to the harmful effects of other environmental factors.

Chemical safety involves the identification of a hazard and a scientific assessment, ideally quantitative, of the risks of exposure, leading on to decisions of the need to manage the risks and, if there is to be management, the nature and extent of the measures to be taken.

Important terms in chemical safety (as defined by IUPAC in 1993) are:

"Risk Assessment: Identification and quantification of the risk resulting from a specific use or occurrence of a chemical or physical agent, taking into account possible harmful effects on individual people or society of using the chemical or physical agent in the amount and manner proposed and all the possible routes of exposure. Quantification ideally requires the establishment of dose-effect and dose-response relationships in likely target individuals and populations".

"Risk Evaluation: Establishment of a qualitative or quantitative relationship between risks and benefits, involving the complex process of determining the significance of the identified hazards and estimated risks to those organisms or people concerned with or affected by them." Risk evaluation, involving socio-economic-political considerations, may be considered an intermediate step between scientific risk assessment and pragmatic risk management.

"Risk Management: Decision-making process involving considerations of political, social, economic, and engineering factors with relevant risk assessments relating to a potential hazard so as to develop, analyse, and compare regulatory options and to select the optimal regulatory response for safety from that hazard. Essentially risk management is the combination of three steps: risk evaluation; emission and exposure control; risk monitoring."

"Exposure assessment: Process of measuring or estimating concentration (or intensity) duration and frequency of exposures to an agent present in the environment or, if estimating hypothetical exposures, that might arise from the release of a substance, or radionuclide, into the environment."

There are a number of approaches to the risk assessment process as delineated by a number of national and international organizations (e.g., NAS, US DHHS, EC, OECD), that have in common the elements of hazard identification, dose(concentration)-response(effect) assessment, exposure assessment, and risk characterization.

- *Hazard identification* is the identification of the adverse effects which a substance has an inherent capacity to cause to humans.

A hazard identification can be based solely on *in vivo* and *in vitro* laboratory data and, where available, toxicity data derived from human observations; structure-activity relationships may also be considered, also included in this step consideration of potential exposure, in terms of production, use, actual or predicted exposure conditions, and exposed populations; if no association with adverse effects was established then a hazard was not identified and the risk assessment process could end at this stage.

- *Dose (concentration)-response (effect) assessment* is the establishment of the relationship between dose or level of exposure to a substance and the incidence and severity of an effect. It is designed to determine the nature and incidence of adverse effects at defined doses(concentrations) in target populations. It is usually based on studies in experimental animals, but where toxicity data derived from observations of human exposure are available they are given special consideration. Animal studies are carried out under controlled laboratory conditions do not usually replicate human exposure and can differ in important variables such as doses(concentrations), patterns of exposure, and age distribution and health status of exposed human populations. Consequently, laboratory data require interpretation and extrapolation for human chemical risk assessment. This interpretation and extrapolation require expert judgement based on experience.

In animal studies important toxicological end-points are the no-observed-adverse-effect level (NOAEL) and, if not available, the lowest-observed-adverse-effect level (LOAEL). These are defined by IUPAC in 1993 as:

NOAEL *The greatest concentration or amount of a substance, found by experiment or observation, which causes no detectable alteration of morphology, functional capacity, growth, development or life span of the target organism under defined conditions of exposure (IUPAC, 1993).* •

LOAEL *The lowest concentration or amount of a substance, found by experiment or observation, which causes an adverse alteration of morphology, functional capacity, growth, development or life span of the target organism distinguishable from normal (control) organisms of the same species and strain under the same defined conditions of exposure (IUPAC, 1993).*

• In 1994 , IPCS added an additional sentence to this definition stating that "Alterations of morphology, functional capacity, growth, development or life span of the target may be detected which are judged not to be adverse."

- *Exposure assessment* is concerned with the determination of the emissions, pathways, and rates of movement of a substance and its transformation in order to estimate the concentrations/doses to which human populations are or may be exposed.

Exposure assessment, whether based on measurement or prediction, thus includes production volume, types and extent of use, exposure routes and media, magnitude and duration of actual or predicted exposures, and individuals, groups, or populations that could be exposed

- *Risk characterization* is the estimation of the incidence and severity of the adverse effects in a human population due to actual or predicted exposure to a substance, and may include quantitative risk assessment(risk estimation).

Risk characterization combines observed toxic effects, their dose-relationships, frequency, and reversibility with human exposure. It can contain a safety evaluation giving a level(s) of exposure that will not result in adverse effects in an exposed population. Risk characterization is the most important component of the risk assessment process for risk management.

Risk management

The broadest possible awareness and reliable assessment of chemical risks is a prerequisite for achieving chemical safety. Subjective perception of the gravity or importance of risk is based on a person's knowledge of different risks and the social, economic and political judgement of their implications. People treat risks differently, depending on whether they are voluntary or involuntary. Risks offensive to the senses are usually treated more seriously. Risk perception is clearly influenced by the risk communication practice, the importance of which is now more and more appreciated (Section 6.1.5). The experts and decision-makers may perceive risk differently from other members of the public, and the risk communication is more effective in the form of a dialogue than as a one-way transfer of facts from experts to the public, and the pressure of requirements expressed by the public on the decision-makers.

The best way to avoid risk is to prevent it. Prevention involves a series of health-oriented activities of various types which may be addressed to entire population, specific risk groups or individuals. Of the many strategies available for the prevention of chemical damage to health, the most effective are those which prevent contact between the toxic agent and humans. These primary prevention strategies, the ultimate objective of which is "zero" exposure or to ensure that levels of pollutants do not exceed the quantity considered safe for health and specified by scientific experts in the risk assessment process, can be realized through both regulatory and non-regulatory measures, such as promotion of the use of cleaner products and technologies, emission inventories, product labelling, use limitations, economic incentives, and the phasing out or banning of toxic chemicals that pose an unreasonable and otherwise unmanageable risk to the environment or human health and those that are toxic, persistent and bio-accumulative and whose use cannot be adequately controlled. Examples of regulatory measures used at various stages of a chemical's cycle are shown below:

Raw material	Industrial process	Product	Waste
Classification and labelling	Exposure limits	Classification and labelling	Recycle
Restrictions of use/ban	Process control Biomonitoring	Restrictions of use/ban	Disposal control
	Emission control (air, water)		

There are often alternatives to toxic chemicals currently in use. Health risk can be reduced by using other less toxic chemicals or even non-chemical technologies. An example in the agricultural area is integrated pest management, when the use of biological control agents is a clear alternative to toxic pesticides. Another example is the use of super critical carbon dioxide instead of chlorinated solvents for cleaning electronic components.

The protection of workers is usually achieved by changing the hazardous process or by engineering controls which can include enclosure of hazardous material/process or improved ventilation. In addition, work practices can be modified, for example by changing the way in which a job is performed so that overall exposure is minimized, by enclosing processes, or by remote control operation. It may be necessary to utilize personal protective equipment to prevent exposure to chemicals in situations where engineering controls or work organization are not sufficiently effective, where exposure conditions are temporary, or where an additional line of defense is necessary. The most widely used personal protective equipment includes respirators, dermal protection, eye/face protection, and hearing protection. It is generally accepted that the use of personal protective equipment should not be the general method of protecting workers, which should be achieved by design and engineering controls, but should be only used when exposure to harmful conditions cannot be controlled effectively by other measures.

Over the last two decades, there has been a growing understanding of the roles of workplace design and workers behaviour in improving worker health and safety. Health and safety education programs can contribute directly to the reduction of exposure and related health effects, e.g., proper use of the personal protection equipment, or indirectly by eliminating specific behaviours contributing to occupational hazards (e.g., smoking, which may increase the risk of cancer for workers exposed to certain carcinogenic substances, or alcohol abuse, which increases the risk of the work-related accidents). Health promotion programs at the workplaces are usually part of the overall health promotion strategy and they promote behaviours related to the general health of employees, such as nutrition, exercise, and stress management. They should also provide necessary conditions and support for employees to comply with the healthy behaviour patterns.

The presence of non-nutrutive chemicals in food and water supplies poses particular problems of safety assurance. Such chemicals include natural toxicants inherent to food organisms, environmental contaminants, residues of pesticides and other agrochemicals, substances migrating into foods and beverages from packaging materials, as well as additives purposefully introduced to achieve particular technological functions. Special considerations arise from the carry over into meat, milk or eggs of residues of veterinary drugs which may also be used in human medicine.

The fraudulent adulteration of foods has been practised for centuries and led many countries to introduce food (and beverage) safety legislation early in the 20th century. Although the legitimate use of food additives was recognized, the responsibility for safety was left to the vendors with little guidance on how safety was to be assured. Apart from the specific prohibition of the addition of certain substances known to be toxic (prohibited lists) and the proscription of addition of any additives to specified basic foods such as milk, there was little regulation of food additive use or guidance from the authorities on acceptable additives. Indeed, the first permitted list of food colours was not introduced in the UK until 1957. Similarly, standards of composition and purity were initially rudimentary and related to a limited number of known toxicants such as lead and arsenic.

The industrialization of food production and processing throughout the 20th century led to an increasing need for guidelines and regulations concerning food additives and contaminants, and for supporting methodology for demonstrating safety or assessing level of risk. The extensive introduction of pesticides into agricultural food production with the inevitable carry over of traces of residues into food (and water supplies) further emphasized this need. At the international level, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) was established in 1956 to establish general principles for ensuring the safety of food additives and to review specific substances currently in use or proposed for use as additives. JECFA has addressed the risks posed by some food contaminants and natural toxicants, and the presence of veterinary residues in food. The issue of the safe use of pesticides and their residues in food has been addressed by a separate committee, the Joint FAO/WHO Meeting on Pesticide Residues (JMPR).

Clearly, the need to assure the safety of foods and beverages led to requirements for the associated toxicological methodology. Since food is consumed over a lifetime, from the neonatal period through growth and development, during the reproductive phase and into old age, the emphasis centred on the effects of chronic exposure to low doses on a daily basis rather than on acute toxicity. Special tests were needed to evaluate effects on reproduction and on the conceptus *in utero*, including effects which might only become manifest in the second generation later in life.

The JECFA has played an important role both in developing principles for the safety assurance of food additives and contaminants and in assessing particular substances. It was material in developing the concept of the Acceptable Daily Intake (ADI) which it defined as that level of a substance which would have no appreciable effect on health when ingested daily over a lifetime. In the related evaluations for contaminants, the concept has been adopted by many states and supranational regulatory

committees, such as the European Scientific Committee for Food. JECFA has developed and refined these principles and these have been reviewed and consolidated in Environmental Health Criteria No. 70 (WHO, 1987). Following the assessment of the toxic risks of food additives, contaminants, and residues of pesticides and veterinary drugs, the outputs (ADI, PTWI, MRL) are used by the Codex Alimentarius and regulatory authorities to ensure food safety. It must be realized that in world trade there can be flexibility in the application of these limits, particularly where strict adherence would compromise food supplies.

As noted in 1999 by IPCS in their publication "Principles for the Assessment of Risks to Human Health from Exposure to Chemicals" (EHC No. 210), "although specific aspects vary, comparable schemes have been developed by various national and international agencies and organizations to derive levels of exposure considered to present minimal or no risk for non-neoplastic effects to the general population. These include: Reference Dose Concentrations (US EPA), Tolerable Daily Intakes/Concentrations (Health Canada), Minimal Risk levels (US ATSDR), Tolerable/Acceptable Daily Intakes (IPCS, 1987a,b, 1994). In evaluating dose-response for non-neoplastic effects, the European Union does not derive tolerable intakes, instead effect levels are compared to estimated exposures ("margins of safety")". The approaches used by these various health organizations share many of the same underlying assumptions, judgements on critical effect and choices of uncertainty (or safety) factors.

Where ADIs cannot be derived, e.g., for carcinogens, the usual recommendation is that exposure be avoided or minimized by the application of good agricultural practice and food technology.

Although zero exposure is usually a desirable goal, it is seldom possible to achieve it. Therefore, exposure limits for chemicals in use are developed and enforced to control unavoidable exposures. Exposure limits occupy a central place in the legislative means relating to the control of toxic chemicals. Their aim is to protect individuals, human population, and their progeny from the adverse effects of hazardous environmental factors.

For establishing health-based exposure limits for noncarcinogens, the lowest-observed-adverse-effect level (LOAEL) and the no-observed-adverse-effect level (NOAEL) are established. Then an appropriate margin of safety is applied (safety factor or uncertainty factor), to allow for the uncertainties of extrapolating from animals to humans (Section 5.4.1). For genotoxic carcinogens, a no-threshold approach may be followed (Sections 2.1.3.3 and 5.4.3) and exposure limits are thus recommended to reduce the risk below a certain value. For non-genotoxic carcinogens, the LOAEL/NOAEL approach (Section 5.4.1) is followed in some countries. The translation of health-based exposure limits into operational regulatory exposure limits or national hygienic standards usually has to take into consideration technological and socioeconomic factors, feasibility and enforcement.

A further approach to ensure that a chemical exposure does not affect the health is targeted health surveillance through medical examinations and biological monitoring. These are both useful additional tools for risk assessment, and can also provide basis for the health protection. Under conditions of the over-exposure, biological monitoring and health surveillance allow of early detection of health effects to undertake appropriate technical and (or) medical remedial actions. However, these methods do not constitute primary prevention of either exposure or health effects.

The global dimension of chemical safety problems call for the global strategy and the comprehensive control approaches. The procedures of registration/notification of chemicals combine risk assessment and risk management issues in one regulatory system. For some types of chemicals e.g., drugs, pesticides such procedures have been in operation for years.

Many countries have regulations requiring the notification of the properties of new and existing chemicals and legal provisions for risk assessment. These require that the effects of new chemicals on human health and the environment must be assessed before the chemicals are manufactured or marketed. Such regulations are well developed in the member states of the European Union. The extent of toxicity testing depends on the volume of a substance placed on the market. Internationally used toxicity testing guidelines have been developed by the Organisation for Economic Co-operation and Development (OECD) for use by member countries to ensure the mutual acceptance of test data (Table 1). In this process they are complemented by Good Laboratory Practice (GLP) principles and procedures.

The introduction of new regulations for testing chemicals and the realization that old toxicity data may require updating led to the view that a considerable amount of work needs to be done to assess the human hazards of existing chemicals . However, the scope of the problem is not always sufficiently recognized, however, the task of evaluating the toxic hazards of existing chemicals is so vast that it clearly exceeds the technical capacity or economic resources available to deal with the problem. Thus, methods of priority setting are essential if resources are to be used most effectively, and the growing national and international activities in the matter should be harmonized to avoid duplication. In 1987, the OECD decided to undertake a systematic investigation of existing chemicals. The programme which had started from high production volume chemicals yields data available worldwide. Greater collaboration is needed in planning approaches to existing chemicals at the international level and between government, industry, trades unions, consumer protection associations, and special interest groups. The European Union Programme came into force on 4 June 1993. This Programme gives a legal requirement that the manufacturer or the importer has to deliver data on the substances produced or imported. The risk assessment process in the EU provides that every member state formally selects priority substances to perform the risk characterization for a priority substance. Exposure assessment and the dose-response are conducted comparing the information on exposure to the effects identified by a hazard identification of the substance. The risk assessor has to decide whether there is or there is no need for further information or testing or whether there is a need for limiting the risks. The draft risk assessment report is sent to the OECD as a European contribution to the Programme on existing substances for discussion with OECD member countries.

Table 1. OECD Guidelines for Testing of Chemicals
Health Effects

Short-Term Toxicology

401 Acute Oral Toxicity
402 Acute Dermal Toxicity
403 Acute Inhalation Toxicity
404 Acute Dermal Irritation/Corrosion
405 Acute Eye Irritation/Corrosion
406 Skin Sensitization
407 Repeated Dose Oral Toxicity - Rodent: 28/14-day
408 Subchronic Oral Toxicity - Rodent: 90-day
409 Subchronic Oral Toxicity - Non-rodent: 90-day
410 Repeated Dose Dermal Toxicity: 21/28-day
411 Subchronic Dermal Toxicity: 90-day
412 Repeated Dose Inhalation Toxicity: 28/14-day
413 Subchronic Inhalation Toxicity: 90-day
414 Teratogenicity
415 One-Generation Reproduction Toxicity
416 Two-Generation Reproduction Toxicity
417 Toxicokinetics
418 Acute Delayed Neurotoxicity of Organophosphorus Substances
419 Sub-chronic Delayed Neurotoxicity of Organophosphorus Substances: 90-day
420 Acute oral toxicity - Fixed Dose Method

Long-Term Toxicology

451 Carcinogenicity Studies
452 Chronic Toxicity Studies
453 Combined Chronic Toxicity/Carcinogenicity Studies

Genetic Toxicity

471 *Salmonella typhimurium*, Reverse Mutation Assay
472 *Escherichia coli*, Reverse Mutation Assay
473 *In vitro* Mammalian Cytogenetic Test
474 Micronucleus Test
475 *In vivo* Mammalian Bone Marrow Cytogenetic Test - Chromosomal Analysis
476 *In vitro* Mammalian Cell Gene Mutation Tests
477 Sex-Linked Recessive Lethal Test in *Drosophila melanogaster*
478 Rodent Dominant Lethal Test
479 *In vitro* Sister Chromatid Exchange Assay in Mammalian Cells
480 *Saccharomyces cerevisiae*, Gene Mutation Assay
481 *Saccharomyces cerevisiae*, Mitotic Recombination Assay
482 DNA Damage and Repair, Unscheduled DNA Synthesis in Mammalian Cells *in vitro*
483 Mammalian Germ Cell Cytogenetic Assay
484 Mouse Spot Test
485 Mouse Heritable Translocation Assay

1.4.1 Universe of chemicals to be tested

The world-wide chemicals industry produced 400 million tonnes of chemicals in 1995 with Europe being the largest chemical producing region in the world accounting for 38% of the total. It is believed that one of the most pressing environmental health priorities for the developed world in the 21st century is posed by the thousands of industrial chemicals for which even the most basic test data are unavailable. It has recently been noted by the US EPA that only 7% of the 3000 high production volume chemicals (HPVC) (chemicals exceeding approximately 500 000 kg/year) currently used in the USA have a comprehensive set of basic health and environmental studies available to the public.

The European Union recently noted that little is known of the toxicity for about 75% of existing chemical substances in current use of the 100 000 listed in the European Inventory of Existing Commercial Chemical Substances (EINECS) data base. While relatively few such chemicals are generally thought to pose a significant risk to human health, safeguarding of public health depends on identifying the effects of these chemicals and the levels at which they become potentially harmful to humans.

The EU initially focused on the hazard assessment of new chemicals introduced after 1981 which were required to have some pre-market toxicity testing. Since 1993 the EU began to assess the risks of the 100 000 existing and 2000 new chemicals that have no limited or adequate toxicity and/or eco-toxicity data, starting with those whose production exceeds 1000 tonnes/year. There are 2500 of these high production volume chemicals which are currently being assessed by the European Chemicals Bureau (ECB) according to the European Environment Agency report in 1998.

It has been noted that for about 75% of the 3000 or so chemicals in large-scale use the "minimal" toxicity data required by the OECD for a preliminary assessment of health hazards to humans are not publicly available. No adequate ecotoxicity data exist for 50–75% of the priority HPVC chemicals reviewed by the EU. The US EPA and the Chemical Manufacturers Association also confirmed that about 75% of chemicals have insufficient toxicity or ecotoxicity data for preliminary OECD risk assessments.

In the USA, the inventory of the Toxic Substances Control Act (TSCA), (administered by the EPA) contains over 70 000 substances and more than 3000 chemicals are added to the inventory annually. Testing or screening all of these chemicals for the many major areas of toxicity, e.g., carcinogenicity, neurotoxicity, reproductive and developmental toxicity, would be very expensive and time consuming, and would additionally involve large number of animals. Hence it is broadly acknowledged that methods are needed to prioritize new and existing chemicals for animal testing. The National Toxicology Program (NTP) in the USA recently noted that there are 85 000 chemicals in commerce today, the impact of many of these chemicals on human health is largely unknown. The NTP can only provide toxicological evaluations (primarily carcinogenicity bioassays) on 10–20 of these chemicals per year.

It should be noted that recently the chemical industry in the USA via the Chemical Industry Institute of Toxicology (CIIT) announced that it would spend one billion

dollars over a 6 year research program that will gather toxicity and other data on nearly 3000 so-called high production volume chemicals that are produced or imported in quantities of more than about 500 000 kg/year. Additionally 15 000 chemical and pesticide ingredients will be screened to determine whether they adversely affect endocrine systems.

1.5 International dimension

Chemical safety has a clear international dimension. Following the lead given by the 1973 United Nations Conference on the Human Environment, held in Stockholm, Sweden, where chemicals were clearly seen as having the potential to damage human health and the natural environment. This international concern about the potential of chemicals to have adverse effects on human health resulted in WHO, together with the United Nations Environment Programme and the International Labour Organisation to cooperate in the International Programme on Chemical Safety (IPCS). The objectives of the IPCS include the evaluation of the effects of chemicals on human health and the environment and the development of methodology and testing methods in order to produce internationally comparable results. Special attention is now given to the risk evaluation of priority chemicals, development of methodology for health risk assessment, technical cooperation, management of chemical emergencies, prevention and treatment of poisonings, and manpower development and training.

In implementing its objectives the IPCS produces many documentary outputs, including the series of Environmental Health Criteria (EHC) monographs that assess the risks of chemicals, groups of chemicals, or physical agents, or describe principles and methods for evaluating toxicity related to systems and organs. Other significant publications include the series of Health and Safety Guides (HSG), Concise International Chemical Assessment Documents (CICAD), Data Sheets on pesticides, Poison Information Monographs (PIM), International Chemical Safety Cards (ICSC) produced in collaboration with the European Commission, and the Safety Evaluation monographs for Food Additives and Contaminants, Pesticide Residues, and Veterinary Drug Residues produced in collaboration with FAO. The EHC monographs on methodology are of particular relevance to the risk assessment process.

The International Agency for Research on Cancer (IARC) also has many publications relevant to chemical safety, including the series of monographs evaluating the carcinogenic risks of chemicals, chemical processes, occupational profiles, and lifestyle, scientific publications on mechanistic aspects of cancer and risk estimation methodology, cancer epidemiology, and cancer research databases.

The implementation of chemical safety, nationally and internationally, has steadily progressed and its importance was fully recognized at the 1993 UN Conference on Environment and Development (UNCED) – the "Earth Summit".

Chemical safety was a high priority issue in ensuring that development would be socially, economically and environmentally sustainable for the twenty-first century. Chapter 19 of Agenda 21, entitled "Environmentally sound management of toxic

chemicals including prevention of illegal international traffic in toxic and dangerous products", calls for a significant strengthening of both national and international efforts to achieve an environmentally sound management of chemicals. At this Conference one of the major topics, concluding in full international agreement, was the environmentally sound management of toxic chemicals, including prevention of illegal international traffic in toxic and dangerous products. The need for a substantial use of chemicals to meet the social and economic goals of the world community has to be balanced against the possibilities of gross chemical contamination, with grave damage to human health, genetic structures and reproductive outcomes, and the environment. Accordingly the UNCED proposed six programme areas:

(a) Expanding and accelerating international assessment of chemical risks;
(b) Harmonization of classification and labelling of chemicals;
(c) Information exchange on toxic chemicals and chemical risks;
(d) Establishment of risk reduction programmes;
(e) Strengthening of national capabilities and capacities for management of chemicals;
(f) Prevention of illegal international traffic in toxic and dangerous products;

The necessity for enhancement of international cooperation was emphasized. The six programme areas depend on this, the application of the technical, scientific, educational, and financial resources, and their coordination. To varying degrees, the programme areas involve hazard assessment (based on the intrinsic properties of chemicals), risk assessment (including assessment of exposure), risk acceptability and risk management.

Recognizing that different countries have different systems for assessing risk and prescribing exposure standards and limits and that this can be a source of confusion it was agreed that greater efforts are required for the development and adoption of consistent approaches. This Conference has provided a new impetus towards the improvement and development of risk assessment methodology with greatly increased international harmonization of approaches and techniques and active cooperation between countries in making and using chemical risk assessments.

UNCED recognized that risk assessment and risk management practices should be closely linked with the good communication between all the partners involved. Risk management decisions related to chemical safety need to be based on scientifically sound risk assessment procedures and risk assessment conclusions should strongly influence or, ideally, govern risk management decisions. However, this relationship can be seriously constrained by the limitations in both availability and quality of data, lack of scientific consensus on their interpretation, difficulties of communication between scientists, decision-markers and the public, and differences in interests and priorities among the various social groups and interests involved. The intensive and efficient international cooperation would definitely strengthen the whole process and promote the exchange of the chemical safety experience and technologies between countries. As a first step, the principles and methods of chemical safety should be clearly understood and harmonized, and procedures unified wherever justified.

The existing effective international collaboration on chemical safety between UNEP, ILO and WHO in the IPCS was considered by the UNCED as the nucleus for international cooperation and environmentally sound management of toxic chemicals. Cooperation with other programmes, such as those of OECD, EU, and other regional and governmental chemical programmes was stressed. Chapter 19 of Agenda 21 stated that all efforts should be made to strengthen the IPCS. Positive outcomes of UNCED have been the establishment of an International Forum on Chemical Safety, which held its first meeting in 1993, and the development of an Expanded IPCS with the addition of the United Nations Industrial Development Organization (UNIDO), the Food and Agriculture Organization (FAO), and the Organisation for Economic Co-operation and Development (OECD) as cooperating organizations, and with close relations with the Commission of the European Union (CEU).

More recent and ongoing developments in IPCS's Harmonization of Approaches to the Assessment of Risk from Exposure to Chemicals should be noted, e.g., the meetings in Lyon in February, 1999 and the meetings of the Steering Committee in October, 1999 in Montreux. Of particular note is IPCS's pursuit of a Conceptual Framework for Cancer Risk Assessment. This framework provides a generic approach to the principles commonly used when evaluating a postulated mode of action for tumour induction by a chemical carcinogen (across all sections such as industrial chemicals, pesticides, food additives), is a key step in the overall process of hazard characterization/risk assessment as well as in the later stage in the overall process - the assessment of relevance to humans. The purpose of the framework is to provide an analytical approach to considering the weight of evidence for a mode of action in a given situation rather than designed to give an absolute answer on what is sufficient information as this will vary depending on the circumstances.

As noted earlier, the achievement of chemical safety is a dynamic, active and continuing process where early recognition of emerging problems, e.g., new types of exposure, toxicities and sub-populations at potential risk require evaluation, analyses and where required national and international monitoring and elaboration. For example, there is growing concern that a number of substances which interfere with the normal functions of the body governed by the endocrine system have the potential of causing adverse effects in humans and wildlife. Endocrine disruptors (EDCs) have been defined by IPCS "as exogenous substances that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny of sub-populations". EDCs encompass a broad spectrum of chemicals including: natural and synthetic hormones, pesticides, persistent environmental pollutants (PCDDs, PCDFs, PCBs), monomers and additives used in plastics. Target organs potentially affected as a result of exposure to EDCs include: male and female reproductive systems, nervous system and thyroid and immune systems.

IPCS and OECD have taken the lead concerning approaches and means for coordinating and supporting efforts to address the issues internationally and coordinating testing and assessment strategies. IPCS has taken the lead on the establishing of a global inventory on ongoing research and the international assessment of the state of the science of EDCs. The IPCS has formed a Steering Group of

15 international scientific experts to provide guidance and technical advice for these activities and this Steering Group has met in Washington, DC, 9-11 December 1998 and 16-18 March, 1998; in Ispra, Italy on 24-26 June 1998; in Stockholm on 21-23 June 1999 and in Washington, DC, on 22-23 November 1999 with the scientific authors to assess the status of the assessment document.

2. PRINCIPLES OF TOXICITY

The development of toxicity in response to the administration of a chemical can be regarded as resulting from two factors.

(1) The sensitivity of the tissue or organ to the effects produced by the chemical on the structure or function of the tissue. The inherent sensitivity of the target organ or tissue depends on the mechanism of toxicity and the ability of the tissue to exhibit that mechanism which may depend on the balance between local activation and cytoprotective reactions. The actions of a chemical at the target tissue are referred to as the **toxicodynamic** properties of the chemical.

(2) The delivery of the chemical to the site of toxicity, which may vary in both magnitude or concentration and in duration. The concentration of the chemical at the target tissue is related to the total body load; therefore changes in body load will affect the exposure of the target tissue. Parameters of critical importance in determining the exposure of the target tissue are the extents and rates of absorption, distribution, metabolism and excretion. These aspects are referred to as the **toxicokinetics** of the chemical.

2.1 Manifestations and mechanisms

2.1.1 Homoeostasis and adaptive changes

Living organisms have a (limited) capacity to respond to environmental variations and stresses, whether physical or chemical, in order to maintain normal function and survival. A simple example of this is Fe^{II}-haemoglobin which in the erythrocyte is continuously undergoing oxidation to Fe^{III}-methaemoglobin but this is reduced back to the Fe^{II} form by methaemoglobin reductases so that circulating levels of methaemoglobin are maintained at low levels and normal oxygen transport is sustained. "Normal" circulating methaemoglobin concentrations depend on the species and what would be considered usual in small laboratory animals may be abnormally high in humans.

Physiological processes are regulated by many hormonal, enzymic and neuronal control systems which may operate at the level of the cell, organ or multiorgan systems. At the cellular level this may be observed as a modulation of enzyme activity by a substrate or, more fundamentally, a modulation of gene expression/transcription and protein synthesis. The observed effect, then, is a change in the level and activity of specific enzymes and/or changes in the subcellular organelles such as proliferation of smooth endoplasmic reticulum. At the level of the organ, the homoeostatic response to stress may be seen as a generalized hypertrophy, or as hypertrophy/hyperplasia or modulation of function of specific cell types such as pancreatic islet cells or renal tubular epithelial cells. In many cases, regulation of organ function is under neuroendocrine control involving hormonal communication between several different

organs. Thus control of renal urinary homoeostasis, via regulation of blood flow, excretory and resorption processes, involves other organs including the adrenal and pituitary. In this manner, in response to environmental changes, normal composition of body fluids may be maintained by regulation of absorptive, metabolic and excretory functions.

These physiological, homoeostatic changes may be considered an adaptation to environmental variations and not all detectable changes in response to a chemical stress are adverse and therefore manifestations of toxicity. However, the adaptive capacity is limited and regulatory mechanisms may become overwhelmed, as illustrated by the oral toxicity of nitrite-induced methaemoglobinaemia. The converse of this observation is that many manifestations of toxicity show a threshold below which there will not be a measurable response. Although it is possible that all toxic manifestations may show a threshold due to homoeostatic and cytoprotective mechanisms, it is controversial whether a threshold exists for genotoxic manifestations including carcinogenesis (see section 2.1.3.3).

In response to exposure to xenobiotic compounds, an organism may attempt to maintain homoeostasis in a number of ways, usually involving facilitation of excretion or, particularly in plants and molluscs but less commonly in higher animals, sequestration within the organism. Typically, excretion of non-polar organic compounds is facilitated by metabolism to polar, water-soluble metabolites. The polar conjugates may then be excreted in urine or bile. Alternatively, as typified by cadmium, the potential toxicant may be sequestered by binding to a metallothionein and stored within cells (ultimately in the kidney in this case) with a very long biological half life. In adaptation by metabolism and excretion, it is not unexpected to see increases in levels of phase I and phase II enzyme activities (section 2.2.4), proliferation of endoplasmic reticulum within the metabolically active cells (e.g. liver parenchymal cells) and, at high dose levels, organ hypertrophy and cell hyperplasia. In sequestration, one might expect to observe increased levels of metallothionein synthesis (not necessarily in the organ where accumulation occurs) and of the potential toxicant in a specific organ/cell type. Within limits of magnitude and duration of exposure, such changes may be considered adaptation but the capacity to adapt may be overwhelmed by large single doses, while sustained exposure may lead to a progression to a pathological condition, e.g., sustained hyperplasia may progress to dysplasia, and neoplasia. This mechanism of "non-genotoxic" tumorigenesis is not infrequently encountered in rodents, notably in liver, kidney or thyroid (see mechanisms of toxicity below). It follows that it is not always readily possible to distinguish between an adaptive and a toxic response in routine toxicity tests and supplementary mechanistic studies may be necessary to determine the no-observed-adverse-effect level (NOAEL). Risk assessment is based on the recognition of hazards or adverse effects and therefore a crucial decision is to differentiate between adverse and non-adverse effects. This decision has to be based on scientific judgement and experience. In recognition that not all effects in a toxicity study may be adverse there has been a trend in recent years to replace the expression no-observed-effect level (NOEL) with the more precise no-observed-adverse-effect level (NOAEL).

Mechanistic research may reveal that early responses, once considered non-adverse, are in fact part of a progressive response of an injured cell or organ, as is now recognized in the case of lead neurotoxicity. In these cases, detection of these early events provides opportunities for intervention to prevent more significant and potentially irreversible changes and frank cellular or target organ damage.

2.1.2 Acute versus chronic exposure

The response of an organism to exposure to a potentially toxic compound will depend on the magnitude and duration of exposure. In this context, acute toxicity has been defined as the adverse effects occurring within a relatively short time of administration of a single dose or multiple doses within a 24 hour period and is concerned mainly with overdose exposure, e.g., to accidental exposures, to drugs, or to certain circumstances of industrial exposure, such as short-duration, relatively high exposure of an operative during application of an agrochemical to a crop or following a chemical spillage or accident. Conversely, chronic toxicity relates to the effects following long-term exposure to sub-acutely toxic doses such as might arise in many industrial situations or exposure through chemicals present in air, water or food.

The responses following these different modes of exposure may differ not only quantitatively but also qualitatively. For example, acute, high doses of nitrite may lead to severe methaemoglobinaemia, cyanosis and death whereas the effects of chronic lower doses are haemolysis and a consequent anaemia. The differences may arise because a high acute dose overloads homoeostatic processes without the ability of the organism to undergo adaptive changes whereas a measure of adaptation may occur following sustained exposure to lower doses, resulting in some degree of acquired tolerance. However, chronic chemical stress may induce changes which are progressive and pathological as indicated above in relation to sustained hyperplasia. Furthermore, if a xenobiotic compound shares a metabolic process with an endogenous substrate, induction of the enzyme(s) involved may produce chronic effects not observable in acute studies. This is the situation where drug-induced osteomalacia follows chronic exposure to anti-convulsants due to induction of cytochrome P-450 enzymes which may be involved in steroid metabolism.

Chemicals with a long biological half- life may accumulate in the tissues such that prolonged exposure to doses which produce no observable acute effect leads to accumulation of toxic concentrations in target tissues. Such is the case with cadmium accumulation in the kidney (half-life = 20–30 years) where the Provisional Tolerable Weekly Intake (PTWI) was based on population critical concentrations in the kidney resulting from long-term exposure. Similarly, the accumulation of polychlorinated biphenyls in body lipids or of canthaxanthin in the retina may take several years to achieve steady state levels because of slow clearance from these tissues

2.1.3 Mechanisms of toxicity

The adverse functional or morphological changes observable in clinical, gross or histopathological examinations are almost invariably a consequence of biochemical

lesions. That is, in general, toxicity arises from interaction with molecular sites leading to derangement of the biochemical processes involved in the normal function and regulation of the cells, tissues, organs and organisms by chemical or physical mechanisms.

Conceptually, in the simplest situation overloading the homoeostatic biochemical processes beyond their capacity to adapt has the potential to cause tissue injury whether the substance involved is a nutrient, endogenous metabolite or xenobiotic. This provides the rationale for thresholded toxicity and the dose, tissue or intracellular levels associated with a breakdown of homoeostasis are open to biochemical investigation, as are the mechanisms involved. As indicated previously, chemical stress may induce detectable biochemical changes which are a fundamental part of the homoeostatic process and it is necessary to distinguish these from toxic responses in order to define a NOAEL. However, in the absence of detailed information on the mechanisms involved in producing a detectable change, it may be difficult to assess whether the change is adverse or adaptive. At this stage, expert judgement should be invoked in interpreting the biological significance of these changes. This judgement clearly is facilitated and rendered more reliable by the availability of data on the mechanisms involved. In the absence of such information, in some cases a default position has been based purely on a statistical appraisal of whether the observed parameters (composition of biological fluids, serum/tissue enzyme levels, organ weights, etc.) lie within the "normal" range for the species. Normal in this context may arbitrarily be taken as within for example two standard deviations of the population mean, but this is clearly a conservative position which would tend to attach toxicological importance to any significant alteration in a clinical parameter.

The various primary biochemical mechanisms which may be involved in provoking a toxic response are shown in Table 2 and are discussed below, with examples. A single chemical may produce toxicity by more than one mechanism. In addition, an action *via* one mechanism may increase toxicity mediated by another mechanism; for example overload of metabolic pathways could cause a disproportionately increased interaction with endogenous receptors and a reversible reaction may trigger an irreversible change. In most cases the detailed mechanism of chemical toxicity is not known and the risk assessment is made in the absence of such data.

Homoeostasis is crucially dependent on the ability of specific enzymes to respond appropriately to a varying flux of substrate. However, there is a limited capacity for the cell to respond to an increased load by increasing enzyme activity through e.g. allosteric regulation or induction of enzyme synthesis. Ultimately, substrate overload occurs such that the flux of substrate exceeds the activity of the enzyme and toxic levels may accumulate within the cell or extracellular fluids. This may occur whether the substrate is of endogenous or exogenous origin, nutrient or anutrient.

The overload of enzymes involved in the metabolism of endogenous nutrients may be observed with substrates such as the amino-acids, phenylalanine, glutamic acid or aspartic acid. Generally, it would be difficult to achieve overloading doses of these amino acids by oral ingestion of protein, except in congenital cases of enzyme

Table 2. Biochemical mechanisms of toxicity

1. Overload of specific enzymes
   (a) involving endogenous substrates
   (b) xenobiotic-metabolizing enzymes
2. Inhibition of specific enzymes
   (a) competitive
   (b) non-competitive
3. Induction of specific enzymes
4. Depletion of protective pools
5. Displacement from carrier proteins
6. Interaction with endogenous receptors
7. Derangement of membrane regulated processes
   (a) ion pumps
   (b) energy-requiring active transport
   © oxidative phosphorylation
8. Genotoxicity

deficiency such as phenylketonuria (phenylalanine hydroxylase deficiency) when toxic levels of phenylpyruvate and phenylacetate metabolites accumulate in blood and cerebrospinal fluid. However, under extreme circumstances, such as administration of large bolus doses by gavage or parenterally of glutamate or aspartate, blood levels may rise from the normal 4–7 μmol/dl to levels in excess of 100 μmol/dl, which appears to be the threshold toxic concentration in the most sensitive species, i.e., neonatal mice. This in turn leads to elevated concentrations in the brain and the manifestations of focal necrotic lesions in the hypothalamus. While the circumstances necessary to produce the neural injury are perhaps not relevant to the circumstances of human exposure to these substances in the diet, the toxicokinetic and mechanistic data were invaluable in the safety evaluation of monosodium glutamate and aspartame as food additives.

Another example of metabolic overload arises in the induction of methaemoglobinaemia by nitrite or some aromatic amines and hydroxylamines. As previously indicated, in normal circumstances there is a background flux of spontaneous oxidation of haemoglobin which is in equilibrium with the reduction of methaemoglobin by NAD(P)H methaemoglobin reductases. The reducing equivalents for the reductase are largely derived from NADPH generated by glucose-6-phosphate in the hexose monophosphate shunt. If the rate of oxidation of haemoglobin is increased by a xenobiotic, this may be matched by an increased rate of reduction at low doses but ultimately, at

higher doses, the capacity of the red cell G-6-PDH to provide the reduced co-factor may be exceeded. As a consequence, with increasing dose above this threshold, levels of circulating methaemoglobin rise, first asymptomatically but ultimately with a consequent hypoxia. The redox state in the erythrocyte is largely buffered by reduced glutathione (GSH) which, on oxidation to GSSG, is reduced back to GSH by a NADPH-dependent glutathione reductase. Chronic methaemoglobinaemia leading to depletion of NADPH disturbs this redox balance and leads to membrane oxidation with haemolysis. In this case, the capacity of the G-6-PDH is overloaded leading to a failure of both of the reductases adequately to maintain homoeostasis. There is considerable genetic variation in the activity of red cell G-6-PDH in humans and hence in sensitivity to chemicals which cause oxidation of haemoglobin or glutathione (as in the condition of favism). Differences such as these have to be considered in deriving acceptable or tolerable intakes and in the risk management procedures adopted.

The capacity of the phase I and/or phase II xenobiotic-metabolizing enzymes to detoxicate exogenous compounds may also be exceeded such that there is a dose-dependent increase in blood/tissue levels beyond the threshold of toxicity. Several of these enzymes are capable of being induced on repeated exposure to the xenobiotic and, in some cases, this forms the basis of increased tolerance and differences between the effects of acute and chronic dosing. In other cases, enzyme induction may lead to increases in toxicity (see section 2.2.4.1).

2.1.3.1 Inhibition of specific enzymes

Many toxic compounds exert their effect by inhibition of specific key enzymes involved in normal cell metabolism, function, regulation or replication. Such enzyme inhibitors may themselves be toxic or, by producing a condition analogous to enzyme deficiency, may increase the toxicity of other substances (possibly endogenous) to which the tissue is exposed. Competitive inhibition may arise when the xenobiotic shares a common pathway with an endogenous substrate leading to an effective overload similar to that described above. An example here is that of co-administration of ethoxyquin which markedly increases the hexobarbital sleeping time by competition for the detoxication pathway. Alternatively, the xenobiotic may lead to non-competitive inhibition which, if irreversible, leads to a reduction in enzyme activity, which is not restored in a concentration dependent way as the inhibitor levels fall but depends on *de novo* enzyme synthesis.

An example of the latter situation is provided by some cholinesterase inhibitors, such as carbamate or organophosphorus pesticides. These inhibitors block the hydrolysis of acetylcholine involved in terminating the neurotransmitter action of acetylcholine in the synaptic nerve endings. Evidence of toxicity may become manifest when cholinesterase activity is reduced to about half of normal while at higher levels of inhibition death results from respiratory failure consequent on neuromuscular paralysis and CNS depression. Toxicity depends on the high affinity of the pseudo substrate (pesticide) for the enzyme and the slow regeneration of the active enzyme, a common feature of many toxic enzyme inhibitors.

Selective enzyme inhibition is also seen with the anti-depressant mono-amine oxidase (MAO) inhibitor drugs. The MAO is involved in the regulation and detoxication of pressor amines, such as tyramine, tryptamine, serotonin and norepinephrine. These amines cause vasoconstriction and hypertension, and there are a number of significant dietary sources for some of these amines, such as mature cheeses, sauerkraut, wines and similar fermented products. Inhibition of the MAOs prevents detoxication, effectively increasing the toxicity of the foods containing the amines leading to an increase in blood pressure and increased the risk of cerebral haemorrhage.

Where inhibition involves an enzyme involved in a hormonal regulatory loop, the consequences may be seen at distant sites and may be profound. For example, the food colour, erythrosine (tetraiodofluorescein) has been shown to inhibit the hepatic deiodinase involved in converting thyroxine (T4) to triiodothyronine (T3) thus modulating the thyroid stimulating hormone (TSH) secretory mechanisms in the pituitary and regulation of thyroid function. As a consequence, the thyroid remains stimulated by TSH and, in the rat, the resultant sustained hyperplasia progresses to neoplasia with the formation of initially benign adenomas and ultimately adenocarcinomas. This sequence of events is prevented by co-administration of T3, confirming the non-genotoxic mechanism of tumorigenesis in this case. Clearly, even though the toxic end-point is carcinogenesis, it is thresholded at tissue levels of erythrosine which are non-inhibitory and have no significant effect on conversion of T4 to T3. Inhibitors of enzymes involved in nucleic acid or protein synthesis, such as 5-fluorouracil (inhibitor of thymidylate synthetase) and cytosine arabinoside (inhibitor of DNA polymerase) are generally cytotoxic but, as might be expected, the rapidly dividing cells of the gut mucosa and bone marrow are particularly sensitive. This sensitivity extends to the developing embryo and these anti-cancer drugs are also teratogenic.

Other examples of specific enzyme inhibition being an important mechanism in toxicity are inhibition of NADH coenzyme Q reductase in the respiratory chain by rotenone, inhibition of cytochrome c oxidase by cyanide, and inhibition of aconitase in the citric acid cycle by fluoroacetate.

2.1.3.2 Induction of specific enzymes

Sustained increased demands on particular enzymes may be followed by induction of enzyme synthesis, often via substrate interactions with receptors involved in regulation of synthesis such as the Ah (dioxin receptor) and PPAR (peroxisome proliferator activated receptor) receptors. In some cases, substrate induction of drug-metabolizing enzymes may be seen as part of the homoeostatic mechanism leading to increased detoxication and this is the case when multiple daily doses of ethoxyquin lead to a reduction in hexobarbital sleeping time (in contrast to the inhibitory effects of co-administration, see above) due to induction of the detoxicating enzymes. However, the fact that the same enzyme(s) may be involved in the metabolism of different xenobiotics and endogenous substrates may lead to interactions between the two processes. As a consequence sustained induction of enzymes involved in metabolism of endogenous substrates may interfere with normal regulatory processes. As mentioned earlier,

prolonged administration of phenobarbitone and some other anticonvulsants results in induction of cytochrome P-450 isoenzymes which are involved in metabolism of steroids, including vitamin D, and a consequent osteomalacia. This results in chronic toxic effects not observed in acute studies. The possibility of interactions of this type also creates problems in defining a NOAEL.

Furthermore, metabolism by some members of the cytochrome P-450 superfamily may result in the formation of metabolites that are more toxic than the parent compound (lethal synthesis or metabolic activation; section 2.2.4.2) and induction of these enzymes may increase the rate of lethal synthesis and hence the toxicity. Thus, treatment of rodents with 3-methylcholanthrene (an inducer of cytochrome P-450 1A or CYP1A) or polychlorinated biphenyls such as Aroclor 1254 (a mixed inducer of CYP1A and CYP2B families) leads to increased metabolism of the food borne pyrolysis products aminoimidazo azaarenes (e.g., IQ, MeIQ, MeIQx) to mutagenic and potentially carcinogenic metabolites.

The gut microflora is an important and adaptable site of foreign compound metabolism and the induction of microbial enzymes may increase the exposure of the host to potentially toxic metabolites, for example the conversion of the sweetener cyclamate to cyclohexylamine, an indirect sympathomimetic amine.

Enzyme induction is in many instances a sensitive parameter of chemical exposure. However, high doses are often needed before chemical disposition and toxicity are significantly altered as a consequence of enzyme induction. One mechanism by which an organism may protect against toxic insult, particularly by electrophiles such as carbonium and nitrenium ions and free radicals as well as reactive oxygen species, is by the presence of endogenous scavengers such as the anti-oxidant vitamins/nutrients. A particularly important component in this process is glutathione (GSH). The role of GSH in protection against oxidative injury in the erythrocyte was mentioned above and it plays a similar pivotal role in other tissues by acting as a reducing agent in the metabolism of hydrogen peroxide and organic peroxides. In addition, GSH serves as a nucleophile which can scavenge electrophilic molecules including those arising from phase I metabolism. These conjugates may be further metabolized to mercapturic acids which are excreted (Fig. 1). During glutathione peroxidase-mediated metabolism of peroxides, GSH acts as an electron donor and is converted to the oxidized disulfide, GSSG which in turn is reduced back to GSH by NADPH-dependent glutathione reductase. Under conditions where the rate of GSH oxidation exceeds the rate of reduction of GSSG, the latter accumulates and, in order to prevent adverse consequences, such as the formation of mixed disulfides with protein thiols, the cell actively excretes GSSG. This can lead to depletion of the GSH pool.

Intracellular depletion of GSH has been associated with the hepatotoxicity of paracetamol. This drug undergoes dose dependent phase II metabolism to a mercapturic acid and conjugates with sulfate and glucuronide. The mercapturic acid is derived from the reactive phase I metabolite, *N*-acetyl-*p*-benzoquinoneimine (NAPQI), which is apparently formed by metabolism involving CYP1A, CYP2A and CYP2B, and is inducible by both 3-methylcholanthrene. At high doses, GSH is depleted and the NAPQI

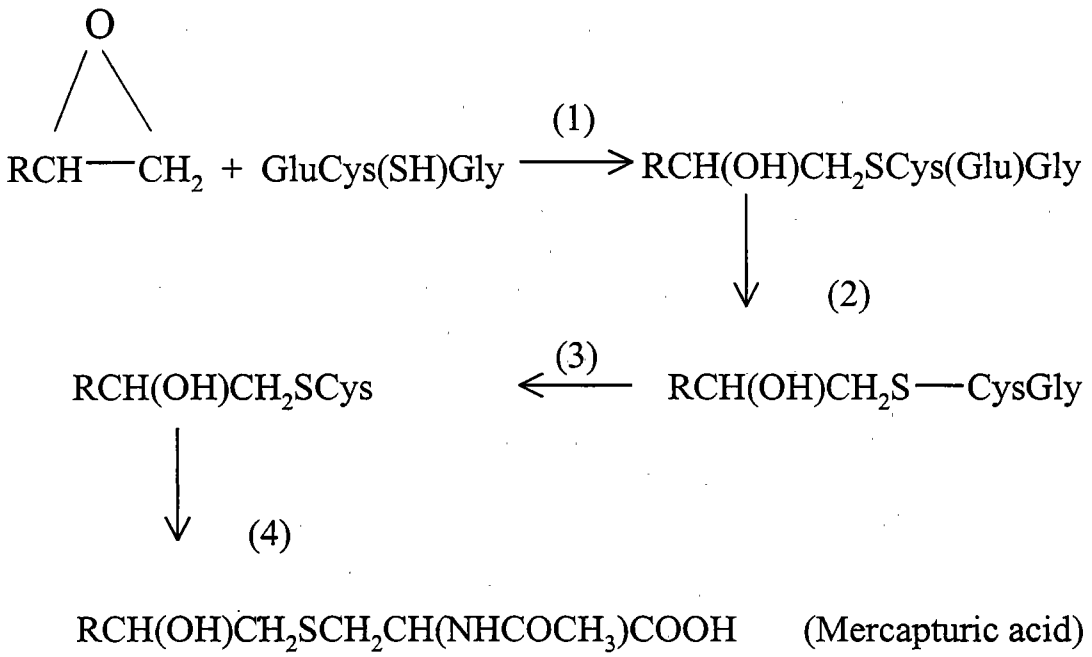

Fig. 1 Mercapturic acid biosynthesis beginning with an electrophilic alkene oxide. Only one positional isomer is depicted for reaction between the epoxide and glutathione, though both would be formed. GluCys(SH)Gly = glutathione (GSH, a tripeptide consisting of glutamic acid, cysteine and glycine).
(1) glutathione *S*-transferase;
(2) gamma-glutamyltranspeptidase;
(3) cysteinylglycinase activity (aminopeptidases);
(4) *N*-acetyltransferase

reactives with other cellular nucleophiles and thus undergoes covalent binding with cellular macromolecules leading to cell necrosis. Other pretreatments which deplete GSH, such as treatment with diethyl maleate, increase the toxicity of paracetamol, further underlining the important role of this protective mechanism.

Many biologically active compounds, both endogenous and xenobiotic, are transported bound non-covalently to specific and non-specific carrier proteins. In this way, pharmacological activity and toxicity may be modulated by sequestration, since it is mainly the free compound which is active. Clearly, saturation of such carrier proteins at high doses may represent a threshold of toxicity above which increasing levels of free compound are available to exert the biological effects. Further, competitive displacement of other endogenous or xenobiotic compounds from carrier protein may occur. The classic example of this toxic phenomenon is neonatal kernicterus following treatment with sulfonamides. The high level of circulating bilirubin common in neonates is largely protein bound but is competitively displaced by sulfonamides with toxic consequences. Where the threshold of toxicity of a single compound may be related to saturation of carrier protein, there is thus the potential for additive or synergistic effects resulting from displacement by a second ligand.

Xenobiotic compounds may exert their toxicity by mimicking endogenous endocrine messengers and binding directly with hormone receptors in responsive tissue. For example, dietary phytoestrogens or synthetic analogues of steroid hormones may have profound effects not only directly on the gonads and reproductive function but on other hormonally responsive tissues such as the uterus, mammary or prostate glands and, indirectly, on body composition. Prolonged stimulation of such hormonally responsive tissues may increase the incidence of tumours although, in some instances the effect may be biphasic. Thus, epidemiological studies indicate that relatively high dietary (not supplemented) levels of phytoestrogens may be protective against mammary cancer, whereas higher, pharmacological doses of estrogens may increase tumour incidence. This may be rationalised in terms of the relative affinity of endogenous and dietary estrogens for the cytosolic estrogen receptors, and the activity of the receptor-estrogen complexes in regulating gene expression. In the particular case of diethylstilboestrol, transplacental effects on embryonic/fetal tissue led to tumours arising in the vagina/cervix of female offspring shortly after reaching sexual maturity. The teratogenicity of retinoids is related to their ability to bind to the cytosolic retinoic acid receptors (RAR).

More subtle effects on behaviour associated with masculinization or feminization may also be a consequence of primary interactions with sex hormone receptors and modulation of endogenous hormone regulation. Certain pesticides, such as methoxychlor (methoxy-DDT) possess estrogenic activity at high doses and affect reproductive function when given to pubertal male or female rats. Long-term exposure causes reduced fertility and increased fetotoxicity and resorptions.

An example of receptor-mediated neurotoxicity is given by lathyrism which is a neurological movement disorder caused by the excessive consumption of the grass-pea (*Lathyrus sativus*). The grass-pea contains toxic amino acids which act at receptors for glutamate, an excitatory neurotransmitter. The principal active toxin is beta-oxalylamino-L-alanine (L-BOAA) which acts stereospecifically via the non-NMDA type of glutamate receptor on the cell membrane.

Many cellular energy-requiring processes are regulated by membrane-bound ion motive ATPases. These enzymes are involved in maintaining intracellular homoeostasis by transferring ions against a concentration/pH gradient and include $(Ca^{2+}+Mg^{2+})$-ATPase, Na^+/K^+-ATPase and the mitochondrial ATP-dependent proton pump.

The importance of the first of this group of enzymes, the "calcium pumps", is evident from the fact that extracellular free Ca^{2+} concentration is about 1–2 mM whereas the internal cytosolic level is about four orders of magnitude less at 0.1 μM. The enzyme is activated by the calcium-calmodulin complex and interference with the calcium pump by specific inhibitors or by oxidative damage to cellular membranes can lead to an increase in cytosolic Ca concentrations. This in turn triggers a number of Ca-dependent biochemical processes which may lead to cell death. Thus several Ca^{2+}-dependent degradative enzymes, including phospholipases, proteases and endonucleases, are activated by a sustained increase in cytosolic free Ca concentration. The activated phospholipases may cause generalized damage to cell membranes, the proteases

(calpains) are associated with loss of integrity of the cytoskeleton and membrane proteins, while the endonucleases play a key role in apoptosis (programmed cell death) (Fig. 2).

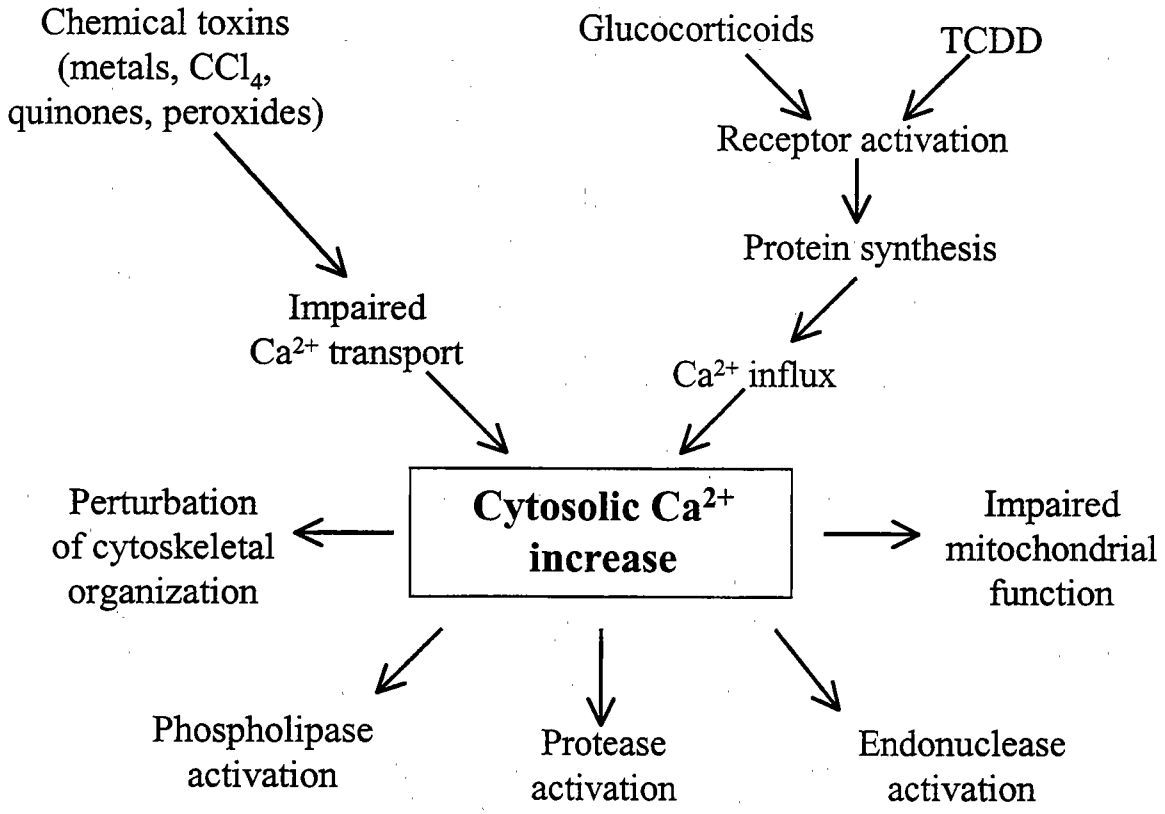

Fig. 2. Activation of Ca^{2+} dependent cytotoxic mechanisms during cell injury and programmed cell death From: Orrenius et al. (1990)

The toxicity of TCDD to thymocytes has been associated with a sustained increase in cytosolic Ca^{2+} levels, endonuclease activation and cell death. Conversely, a block of the endonucleases e.g. by phorbol esters, which activate protein kinase C (PKC), leads to cell proliferation and it is postulated that PKC activation is one mechanism by which Ca^{2+}-dependent endonuclease activation is blocked in stimulated or dividing cells.

The Na^+/K^+-ATPase, or sodium pump, maintains a relatively low cytosolic Na^+ and high K^+ concentration against the extracellular environment. It is involved in maintaining a transmembrane electrochemical gradient which is a primary source of energy for the active transport of various nutrients and for the action potentials of excitable tissues such as muscle and nerve. A number of pesticides, including DDT and pyrethroids, exert their toxicity by effects on the sodium pump.

The mitochondrial electron transport systems involved in oxidative phosphorylation are coupled to ATP synthesis by a proton gradient across the mitochondrial inner membrane involving a total of four proton pumps. Compounds which act directly on these pumps or on the integrity of the mitochondrial membrane may thus block the coupled ATP-synthesis, leading to cell death. Many of the pathological, cytotoxic features of oxidative injury appear to be mediated by effects on membrane integrity and the functionality of these processes of maintenance of intracellular ion homoeostasis.

Many depressants of the central nervous system (such as some gases and organic solvent vapours) may act through general membrane perturbation of neuronal cells.

2.1.3.3 Genotoxicity and carcinogenicity

Genotoxicity is an important toxic end-point which may be associated with somatic mutation, germ cell mutation, teratogenicity, carcinogenicity and acute cell death.

The induction of germ cell mutations, which may continue to affect future generations, is an important consideration in the protection of human health. Current genotoxicity tests are aimed largely at detecting somatic cell mutations and are used as predictive indicators of carcinogenic potential. Relatively few genotoxic agents have been demonstrated to affect germ cells *in vivo*. Genotoxicity is an important factor in the action of some, but not all, teratogenic chemicals. There are increasing reports that certain diseases of ageing are associated with specific genetic abnormalities and it is possible, but not established, that the incidence of these conditions may be dependent on genotoxic exposures during life as well as to innate genetic traits. Also, acute cell death leading to organ necrosis may be initiated by a genotoxic action, for example by some alkylating agents.

Carcinogenesis may be regarded as a process consisting of several stages: an induction of relatively specific genetic damage, i.e., mutation or clastogenesis, which initiates the cell to cancerous behaviour, which is followed by promotional cell replication to expand the clones of initiated cells and then progression, further genetic events and cell replication that leads to tumours of increasing malignancy (Fig. 3a and 3b). The number of specific genetic changes required to induce malignancy varies from tumour to tumour. For example, only two mutations (one in the germ line) are necessary for the development of bilateral retinoblastoma of childhood, whereas four mutations, one in an oncogene and three in tumour suppressor genes, are often found in the most malignant colorectal cancers.

To date four different ways in which chemicals may lead to cancer have been identified, each of which is compatible with Cohen and Ellwein's 1991 hypothesis. The best recognized of these mechanisms is the generation of electrophiles during the course of metabolism of the test compound that interact with DNA. The DNA adducts formed in this way will lead to genetic lesions if not repaired before DNA or cell replication occurs. More recently attention has been focused on the ability of certain synthetic or naturally occurring chemicals to induce oxidative DNA damage, of which there are about 20 different types. Oxidative DNA damage is an ongoing process which normally

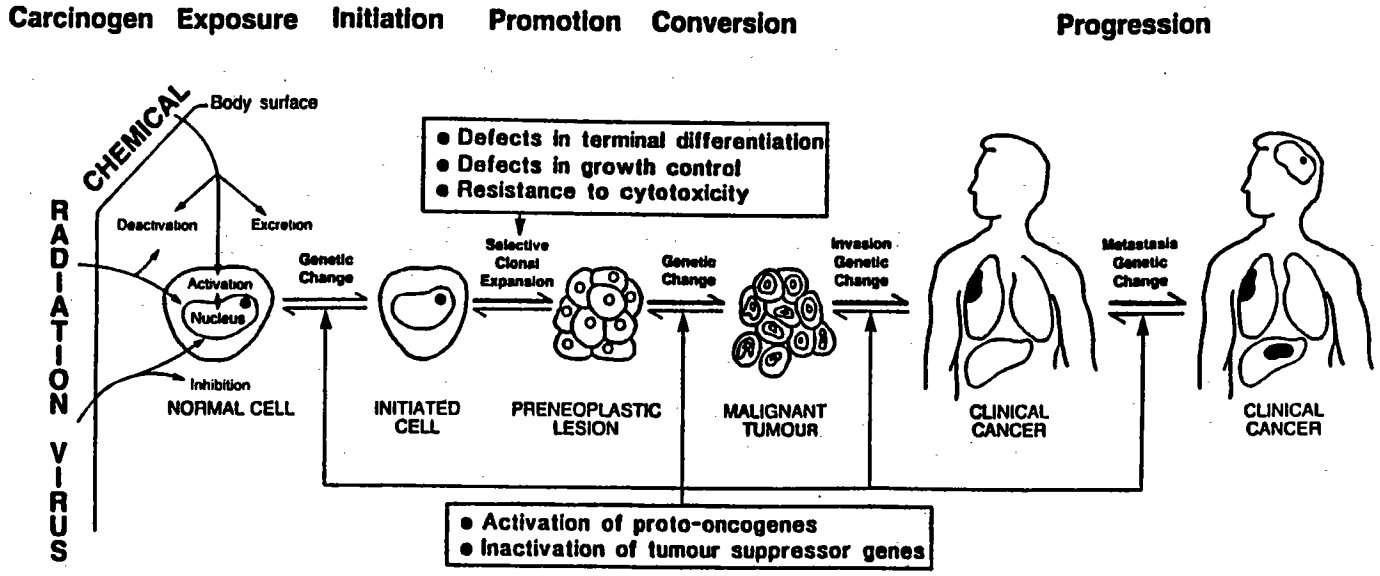

Fig. 3a. Carcinogenesis is a multistage process involving multiple genetic and epigenetic events in proto-oncogenes, tumour suppressor genes, and antimetastasis genes.

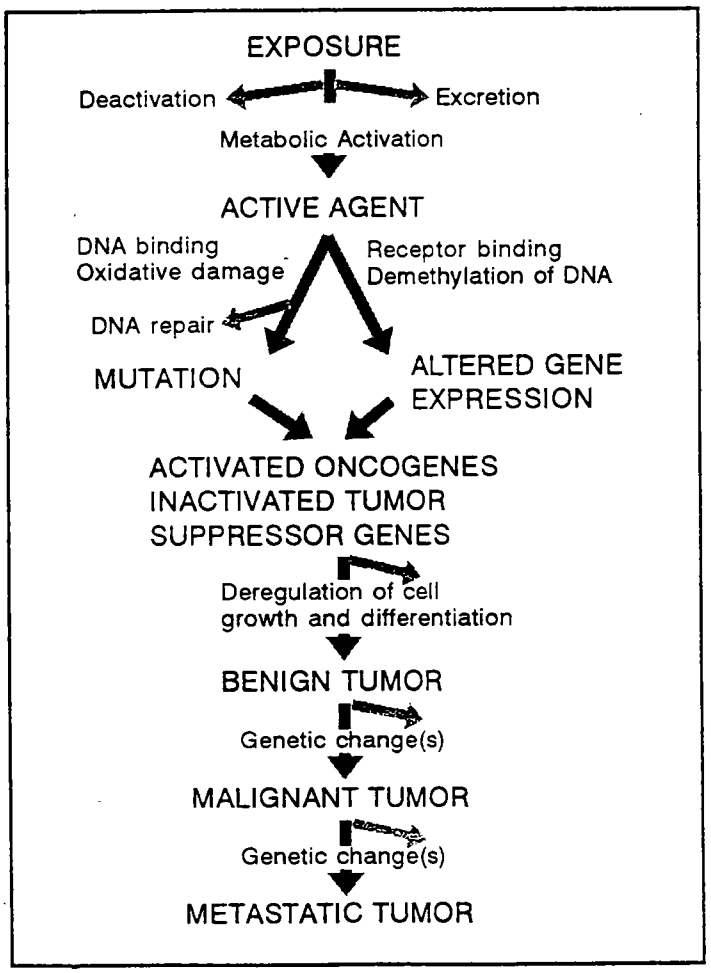

Fig. 3b. Paradigm of cancer development

reaches high levels and must therefore be regarded as being very efficiently repaired. It is possible that a detectable carcinogenic effect due to DNA oxidation may require the overwhelming of the DNA repair mechanism. The third carcinogenic mechanism is the interaction of the chemical with certain receptor proteins that modify the functioning of specific critical sites in the DNA and lead to important changes in cell behaviour, such as apoptosis, differentiation or cell division. This mechanism has been proposed recently for peroxisome proliferators many of which cause rat liver carcinogenesis. Finally it is possible for chemicals to act as carcinogens through their ability to enhance the incidence of tumours, in some cases primarily by producing an increase in mitosis, such as after prolonged cytotoxic damage and regeneration by nitrilotriacetic acid (NTA) or chloroform thereby promoting any spontaneous genetic damage, or increasing the probability of fixation of DNA damage as a gene mutation. Different dose-response relationships may occur as a consequence of these different mechanisms.

It is commonly asserted that mutagenesis by reactive electrophiles is a purely stochastic process and that therefore there would not be a threshold of exposure below which an effect would not occur. Although theoretically thresholds may exist for all mechanisms of carcinogenesis due to repair and homoeostatic mechanisms, it is possible that for genotoxic chemicals the threshold may be at such low exposures that it is not relevant to risk assessment. In the absence of strong evidence to the contrary it is often considered prudent to assume that genotoxicity does not show a threshold.

In contrast, it is likely that a biological threshold exists for other mechanisms and that there would be a level of exposure at which no effect would be produced due to homoeostatic, cytoprotective and repair processes. For example, it is generally accepted that high doses of chemicals which result in cell killing can increase the tumour incidence due to the increased opportunity for carcinogenic mutations to occur because of the increased number of cell divisions required to replace cells; since thresholds are believed to exist for cytotoxicity, it is logical to consider that they would occur also for tumorigenicity by this mechanism. The presence or absence of a threshold for non-genotoxic carcinogens cannot be demonstrated on the basis of dose-response data and mechanistic data are necessary to establish the likelihood of a threshold. At present there are a few examples only where such data are considered adequate evidence, e.g., bladder tumours associated with stone formation. Non-genotoxic mechanisms may be involved also in the generation of some species and sex specific tumours such as the α-2μ globulin dependent renal tumours in the male rat e.g. d-limonene and unleaded gasoline. Mechanistic information has contributed significantly to the evaluation of rodent forestomach tumours induced by BHA or propionic acid, to rodent thyroid tumours following treatment with erythrosine (see above) or ethylene thiourea (ETU) and to bladder tumours in male rats exposed to sodium saccharin, sodium ascorbate or sodium erythorbate. Similarly, a non-genotoxic mechanism of questionable relevance to man is probably involved in the tumours consequent on peroxisome proliferation in rodents.

In practice frequently the only mechanistic data available for rodent carcinogens is the presence or absence of genotoxicity and this is often the factor which is used as the basis for deciding whether a threshold is likely or not and therefore which risk assessment procedure should be adopted (see section 5.4). This pragmatic approach is scientifically questionable because of the lack of correlation between the presence or absence of genotoxicity and the shape of the dose-response curve. In addition some mechanisms of carcinogenesis such as the receptor mediated type cannot be classified readily as threshold or non-threshold: for example with peroxisome proliferation the presence or absence of a threshold may depend on whether receptor occupancy is sufficient to produce a response in the absence of exposure to the chemical. Low dose linearity is to be expected when endogenous and other exogenous factors are sufficient to produce spontaneous tumours and a chemical augments this process. Hence a non-genotoxic chemical may or may not exhibit a threshold depending on the situation. Nevertheless the use of a threshold approach for non-cancer end-points and for non-genotoxic carcinogens and a non-threshold approach for genotoxic carcinogens (section 5.4) is probably the best default approach at present and may be defensible in many situations but only after analysing the totality of the available data. These default approaches should be used with caution, realizing that they are only justifiable in the

absence of relevant data on the toxicological mechanism and that they arose from practice rather than from theory and experimental data.

2.1.4 Local versus systemic effects; routes of exposure

In some toxicity studies, only local effects may be observed at the site of exposure and which may be concentration rather than dose dependent. These local effects may be interpreted somewhat differently than systemic effects in distant tissues/organ systems. However, it is possible that effects may be produced at a distal site which are an indirect consequence of a local toxic effect at the site of administration and are not a direct effect of the chemical on the distal site. Thus, in the toxicological evaluation of orally administered sulfiting agents in rodents and pigs, the first effect observed was an irritant effect in the glandular stomach. At higher doses (dietary concentrations) the inflammatory response showed a dose related increase in severity leading ultimately to ulceration and gastric haemorrhage. At these extreme dose levels, secondary effects of enlargement of the spleen, increased formation of blood cells and anaemia were observed, but these were not considered to represent systemic toxicity. In the light of the high tissue (particularly liver) levels of the detoxicating enzyme, sulfite oxidase, the ADI was based on the NOAEL for the local irritant effect. However, this ADI (0.7 mg/kg body weight) may be exceeded by a significant proportion of the population (notably wine or beer drinkers) but concentrations in the major sources are below irritant levels and, in the absence of systemic toxicity, it has not been necessary to take extreme regulatory measures to ensure that the ADI is not exceeded. For other reasons, notably the existence of a highly sensitive sub-population of asthmatics, strict labelling requirements have been introduced in some countries.

2.1.5 Primary and secondary effects

As indicated above, a primary toxic effect such as gastric irritation and haemorrhagic erosions may lead to secondary effects like increased haemopoietic activity, splenomegaly, and, over a period of time, anaemia. In the case cited, the secondary effects were consequent on blood loss and not a direct effect of the treatment on the blood forming elements, i.e., they were secondary to a local effect and not a direct manifestation of a systemic toxicity.

Secondary effects of this type are not uncommon in toxicity studies and, in some situations, may mask the primary event thus making interpretation difficult without resort to *ad hoc* mechanistic studies. These difficulties become particularly serious when the secondary effect observed is (non-genotoxic) carcinogenesis or tumour promotion. The following cases serve to exemplify the complications arising from serious secondary effects.

Administration of high dietary doses of poorly absorbed, osmotically active materials to rats frequently leads to an enlargement of the caecum. This may be seen with substances as disparate as chemically modified starches, lactose, some polyols (e.g. sorbitol, xylitol) polar food colours and even magnesium sulfate. It has been proposed that this represents an adaptive response to maintain normal osmolality in the lower

gastrointestinal tract. However, caecal enlargement in the rat is quite commonly associated with nephrocalcinosis, a condition also associated with an imbalance of the dietary Ca:P ratio and compounded by magnesium deficiency. Normal calcium homoeostasis is regulated by vitamin D which must first undergo two hydroxylation steps to 1,25-dihydroxy-vitamin D before it can function on the target tissues of the gut (regulating absorption), bone (controlling mineralization/mobilization) and kidney (modulating resorption). The first hydroxylation step occurs in the liver, forming 25-hydroxy-vitamin D which is transported bound to a carrier protein to the kidney where 1-hydroxylation takes place. Control of the 1-hydroxylation step is effected via the trophic effect of parathyroid hormone (PTH); hypocalcaemia leads to increased PTH secretion which increases renal 25-hydroxy-vitamin D 1-hydroxylase activity. In turn, the 1,25-dihydroxy vitamin D functions like a hormone in modulating expression of calcium binding protein, absorption of calcium in the gut, mobilization of bone calcium. In the case of caecal enlargement, this homoeostatic mechanism may be deranged since facilitated absorption may occur by a process which is not regulated by vitamin D and the resultant incipient hypercalcaemia may be compensated by renal excretion, leading to the solubility product of sparingly soluble calcium salts being exceeded and precipitation in the kidney i.e. nephrocalcinosis. Thus a primary overload of hydrolytic or absorptive processes in the gut can produce secondary pathology in the kidney. Even more remote secondary pathology has been observed with some polyols where adrenal medullary hyperplasia and neoplasia (phaeochromocytoma) have been reported, a situation resembling adrenal changes associated with hyperparathyroidism.

A further example of secondary effects is given by the case of erythrosine, mentioned earlier, where the primary event is inhibition of the hepatic deiodination of T4 to T3 as a result of which the negative feedback loop in regulation of thyroid function is impaired. In consequence, the thyroid is maintained in a stimulated state by TSH resulting in hyperplasia. In the rodent, a secondary consequence is an increased incidence of thyroid adenomas and adenocarcinomas.

Numerous other examples of secondary effects could be cited and a clear understanding of the primary event leading to perhaps more obvious secondary sequelae is of importance in interpretation of toxicological observations.

2.1.6 Target organs

Most chemicals display a measure of organ selectivity (organotropism) in relation to the organs/tissues in which they produce their toxic effect. However, the target organs may differ with the species, the route and/or mode of administration (acute or chronic) and with concentration and/or dose. Specific tissues may become targets for toxicity because they are the local site of administration, because they are involved in the accumulation, metabolism or excretion of the chemical or because they show a particular sensitivity to the actions of the chemical or its metabolites. The sites at which toxic effects are most commonly observed are shown in Table 3.

Table 3. Common target sites at which toxic effects are observed

SITE OF EXPOSURE
Skin
Eye
Nasal epithelium
Lung
Gastrointestinal tract
Injection site
SITE OF METABOLISM
Gastrointestinal tract (inc. gut microflora)
Liver
Lung
Kidney
Sites of specialized metabolic activity
SITE OF ACCUMULATION
Liver
Kidney
Retina
SITE OF EXCRETION
Liver
Gastrointestinal tract
Kidney
Bladder
SITE OF SPECIAL SENSITIVITY
Gonads
Secondary sex organs
Conceptus
Nervous systems
Neuro-endocrine organs (e.g., thyroid, adrenal, pituitary)
Haemopoietic tissues (liver, spleen)
Immune system (thymus, lymph nodes) Rapidly dividing tissues (e.g. gut mucosa, bone marrow).

2.1.6.1 Site of exposure

Adverse effects may be observed at the site of exposure to foreign substances as this is the site exposed to the highest initial concentrations. Where the site of exposure is

not also a major site of metabolism, the effects of acute exposure may be limited to local irritation or corrosive effects. Thus, in inhalation studies, the effects may be seen as local inflammatory reactions in the nasal and bronchial epithelium (and possibly conjunctivae). Similarly, topical exposure may lead to dermatitis, dietary or gavage exposure may lead to gastric irritation, and there may be local effects at injection sites of substances administered parenterally.

Local non-systemic reactions which are not dependent on metabolic activation are frequently dependent on the profile of exposure during the administration of the chemical. Effects may be dependent of transient exposures to very high local concentrations, such as would occur with bolus dose administration, rather than on the total dose administered over a more prolonged period. Thus the same total dose may be irritant or even corrosive at high local concentrations but well tolerated at the lower concentrations which would arise from a larger surface area for administration or from a more sustained exposure to lower concentrations. This clearly needs to be taken into account in the design and interpretation of toxicological studies and in the light of the purpose of these studies and the circumstances of human exposure. Under such circumstances it would be more logical to base the risk assessment on the concentration administered rather than the total dose. This is also a consideration in risk management. An obvious but telling example is that of sulfuric acid where a small amount of the pure substance may cause local injury whereas a much larger dose as a dilute solution may be without effect. However, more subtle concentration-dependent effects may be less readily distinguished from dose-dependent phenomena and some examples are discussed later.

Although local effects at the site of exposure may be a result of physical rather than chemical insult, the consequences of either acute or chronic exposure may be serious, even life-threatening, and cannot simply be discounted. Acute, high dose, exposure may lead to serious impairment of organ function (e.g. blinding, respiratory distress, emesis) whereas chronic lower dose irritation may lead to a consequential fibrosis or to the progression from sustained hyperplasia to dysplasia/neoplasia. There are numerous examples to illustrate these sequences of events in substances which are not systemically toxic or carcinogenic but which may be either at the site of exposure.

Chronic chemical or physical injury to the nasal tissues is associated with a number of industrial diseases such as the nasal tumours arising in woodworkers and leather workers chronically exposed to wood dust by inhalation. In experimental inhalation studies, particularly in obligate nose breathers, the nasal tissues are the first site of exposure and may also be subjected to chronic injury. For example, in inhalation studies on formaldehyde in rodents, tumours appeared in the nasal turbinates, the prime site of exposure.

Lung damage on exposure to chemical or physical trauma is a common feature of inhalation studies on dusts and vapours. The site and nature of the response to dusts and aerosols varies with the particle size and physical form (see Section 2.2); the former determines the degree of penetration and site of deposition in the airways while the latter may influence the nature of the response, as exemplified by the response to

talc/asbestos dust. The effects of chronic exposure to such local irritants may be evident as fibrosis and loss of respiratory function, as in chronic bronchitis, silicosis; alternatively, as in asbestosis, the physical characteristics may lead to continuous tissue insult, cell death and sustained mitosis, progressing to the characteristic mesothelioma.

In some cases, the respiratory system is not only a site of exposure but also a site of particular sensitivity or idiosyncratic hyper-reactivity, and there may be serious adverse reactions to irritant gases such as ozone, nitrogen oxides or sulfur dioxide. For example, some asthmatics may react to such irritants with severe bronchoconstriction and fatalities have resulted from this mechanism. Thus, the inhalation of vapours above foods preserved with sulfur dioxide has been implicated in provoking such attacks.

Sulfur dioxide and sulfiting agents provide a good example of compounds which have a low systemic toxicity (due to rapid detoxication by metabolism to sulfate) but which are local irritants at high concentrations. This irritant effect is not only seen after inhalation but also on oral administration of sulfiting agents (sodium metabisulfite) at high dietary concentrations. At concentrations up to about 1% no effects were observed, but above this level inflammatory reactions were observable in the glandular stomach, increasing in severity with concentration from 2 to 8%, ultimately causing gastric ulceration and haemorrhage. Clearly, the concentrations required to produce these local effects are extremely high relative to the exposure of humans from food preservative use of sulfiting agents and this needs to be borne in mind in characterizing the hazard and assessing the risk from such use. The forestomach of rodents is another primary site of exposure to high concentrations of substances administered in the diet or by gavage and local effects at this site have complicated the safety evaluation of a number of orally administered compounds, notably the food anti-oxidant BHA and the preservative, propionic acid. Both of these compounds cause forestomach tumours when administered at high dietary concentrations of about 1–2%. In fact, with BHA dose-dependent (i.e. concentration-dependent) changes were observed from dietary concentrations of about 0.25% upwards in the form of hyperplasia and hyperkeratosis of the squamous epithelium, progressing to benign papillomas at about 1% and frankly malignant carcinoma above this concentration. Thus, in the forestomach as with the nasal epithelium and lung, sustained local irritation and hyperplasia may progress to neoplasia by a non-genotoxic mechanism (see above). In the evaluation of BHA, some regulatory authorities have concluded that, since the neoplasia is consequential on sustained hyperplasia, an NOAEL for forestomach hyperplasia may form the basis for establishing an Acceptable Daily Intake (ADI).

For poorly absorbed compounds, the lower gut may also represent a site of exposure to relatively high concentrations of the orally-administered test substance. In these circumstances, changes may be seen as a direct consequence of these high concentrations or as a result of effects on the symbiotic relationship with the gastro-intestinal microflora. A very common manifestation in rodents is caecal enlargement due to osmotic effects and which has been observed with a wide range of compounds, organic and inorganic, from magnesium sulfate to a number of poorly digested carbohydrates and some polar food colours. High concentrations of such substances may also induce an osmotic diarrhoea. This again represents a non-systemic, concentration-

dependent effect although, as indicated earlier, there may be secondary sequelae to gross caecal enlargement affecting renal and adrenal pathology.

Finally, local effects may be seen at the site of injection of parenterally administered compounds. Irritant compounds may produce an inflammatory response, e.g., peritonitis on i.p. administration or local granulomata on subcutaneous injection. Again, chronic administration may lead to proliferative changes progressing to neoplastic lesions. Thus repeated subcutaneous injection at the same site of some triphenylmethane food colours gave rise to injection site sarcomas by a mechanism which was concentration-dependent and related to the surface activity of the solutions injected; no such effects were seen with the same doses administered at different sites on each successive day.

While several of the examples cited are of local effects which are independent of metabolism or systemic toxicity of the test substance, not all effects seen at the sites of exposure are of this type since the sites of exposure may also be sites of metabolism. For example, there may be significant metabolism of xenobiotic compounds in the Clara cells of the lung or the mucosal cells of the gastro-intestinal tract. Nevertheless, the combination of metabolic activity and high exposure may make these the target organs.

Furthermore, some highly toxic compounds which are inherently active and do not require metabolic activation may produce effects in almost any tissue with which they come into contact and this may make the site of exposure a particular target because of the high dose received at that site. For example, some nitrosamides (unlike nitrosamines) are direct acting carcinogens without a requirement for metabolic activation and consequently do not show the characteristic organotropism of nitrosamines. The route of exposure of such compounds is thus a major determinant of the target organ(s) affected.

2.1.6.2 Site of metabolism

The target organ may represent the site of metabolism through a number of different mechanisms. Firstly, and probably most significantly, many xenobiotic compounds require metabolic activation to the proximate toxic metabolite (see section 2.2.4.2). If such a metabolite is a highly reactive electrophilic molecule or radical, it will have limited opportunity to diffuse away from the site of formation; the effect will be seen in the tissue and even the cell in which metabolism occurs. Secondly, in undergoing metabolism, the compound may deplete the cell of substrates with an important role in maintaining cellular homoeostasis, effectively limiting the capacity of some endogenous biochemical pathways. Thus, competition for reducing equivalents like NAD(P)H, or depletion of glutathione may render the cell prone to oxidative damage. Thirdly, the xenobiotic may mimic an endogenous substrate so that metabolism and effect are directly linked. An example of this is provided by the organophosphorus pesticides where anticholinesterase activity is consequent on the compound mimicking acetylcholine as a substrate (see section 2.1.5).

Organotropism (organ selectivity) may arise from one or more of these mechanisms and this may vary also with species. The organotropism demonstrated by nitrosamines provides a good example of the link between metabolism and site of action, and involves a number of organs, notably liver, kidney, lung and oesophagus. Dimethylnitrosamine (N-nitrosodimethylamine, NDMA) requires metabolic activation by C-hydroxylation with subsequent dealkylation and formation of the methyldiazonium ion; the C-hydroxylation is carried out by cytochrome P-450 2E1 (CYP2E1) and the location and activity of this enzyme largely determine the dose- and species-dependent organotropism. Thus, at low oral doses, metabolism is complete on a first pass through the liver which is the target organ in the rat. At higher doses in which first-pass metabolism is incomplete, tumours appear at other sites, notably the kidney. The lung as a target organ is more sensitive in the hamster than the rat, and this correlates with the relative activities of CYP2E1 in these species.

Within a target organ, specific cell types are commonly the target for toxic injury as they are those involved in metabolism of the xenobiotic. The pulmonary mycotoxin, 4-ipomeanol causes lung damage (oedema, congestion, haemorrhage) in several test species even when given intraperitoneally. The primary injury appears to be cell necrosis specific to the bronchial Clara cells. In these cells, but not the ciliated cells of the lung, activation to an alkylating species takes place with subsequent covalent binding. Although the cytochrome P-450 involved in metabolic activation is also present in the liver in greater quantity, the maximum rate for covalent binding in the specific lung cells is higher than the liver and this provides a rationale for the organ and cell specificity.

The dependence of metabolic activation on tissue/species specific forms of activating enzymes, such as those of the cytochrome P-450 superfamily, indicates the potentially valuable role of metabolism studies in elucidating the relevance to man of effects seen in test animals. *In vitro* studies on genetically modified cells e.g. hepatocytes carrying specific human cytochrome P-450 genes, may help to clarify the role of species specific enzymes in metabolic activation and the significance for humans.

Sites of specialized metabolic activity may be the targets for "overdose" toxicity by endogenous substrates or closely related analogues. For example, focal necrotic hypothalamic lesions are induced, particularly in neonatal rodents, by high parenteral doses of glutamate which is an excitatory neurotransmitter in the central nervous system.

2.1.6.3 Site of accumulation

In some instances, the target organ is the site of accumulation; at this site the tissue concentrations first achieve toxic levels. Examples are provided by the toxicity of a number of metals.

Chronic iron overload, as has been reported among the Bantu of South Africa, results in a high incidence of tissue deposition of iron (siderosis). In severe cases, this

can lead to accumulation of toxic doses in the liver with fibrosis and cirrhosis, as well as deposition of iron in the pancreas, adrenals, thyroid, pituitary and heart. Similarly, on chronic exposure, copper accumulates in the liver with insidious onset of hepatic necrosis (cell death) and cirrhosis. In the case of cadmium, protein binding to a metallothionein first occurs in the liver but translocation to the kidney ultimately leads to localization in the tubular cells bound to another tissue-specific metallothionein. The bound form is effectively not excreted and the biological half-life is extremely long (20–30 years in humans). As a consequence, low dose exposure over prolonged periods can lead to the accumulation of toxic levels in the kidney tubules and a characteristic microglobulinuria. Secondary to the kidney damage is a renal-dependent skeletal deformities (osteomalacia) as occurred in Itai-Itai disease.

Many lipophilic organic compounds tend to accumulate in adipose tissue. Since such substances commonly require metabolic activation to exert a toxic effect and adipose tissue is relatively inert in this regard, such accumulation may be asymptomatic. However, the build-up of such compounds in membrane lipids may have toxicological sequelae. Thus long-term dietary exposure to brominated vegetable oils resulted in interference with cell membrane function and cardiac myopathy; fasting may increase toxicity due to the release of lipid soluble chemicals which accumulate in adipocytes. Similarly, localization of solvents in lipid-rich nerve tissue can result both in acute intoxication and chronic neuropathies.

Many organic solvents exhibit appreciable volatility and have the potential on acute high-level vapour exposure to cause central nervous system depression and narcosis. It has been proposed that these effects result from a physical interaction with neuronal cells leading to general membrane perturbation. However, solvent effects on the central nervous system may be due to more specific interactions with receptors in brain cells. In addition, some solvents such as n-hexane and 2-hexanone (methyl n-butyl ketone, MBK), may cause neuropathies through more specific mechanisms.

The phospholipidoses observed particularly in lungs and adrenals on treatment with some amphiphilic drugs, such as chlorphentermine, have been associated with localization in the lipids of these target organs but not the liver.

Accumulation may occur at rather unusual and compound-specific sites as typified by the carotenoid, canthaxanthin, which has been used as a drug in the treatment of light sensitive psoriasis and as a food additive. Following therapeutic use, deposition occurred in the retina resulting in a characteristic "gold-dust retinopathy". In such cases, the biological half-life in the target tissue may be a major consideration in the safety evaluation process.

2.1.6.4 Sites of excretion

Pathology at the site of excretion is a fairly frequent observation in toxicity studies. Independently of the function of excretory organs in the metabolism of xenobiotics (see above), this may result from the fact that the compound or a metabolite is concentrated in urine or bile with consequent effects on the bile duct epithelium, kidney tubules or

urothelium. The particular mechanisms are not always clear and may be species and sex specific, as in the case of bladder tumours induced in male rats by sodium saccharin. This non-genotoxic carcinogenesis at high doses may be consequential on sustained hyperplasia related to the formation of microcrystals. Similarly, proliferative changes in the bile duct epithelium may progress from hyperplasia to neoplasia by non-genotoxic mechanisms (e.g. coumarin).

Further site-specific effects relating to the function of the excretory organs may be secondary to changes in the composition of urine or bile. For example, ethylene glycol is oxidized to oxalic acid and, in association with calcium ion in the urine, the solubility product may be exceeded leading to precipitation of calcium oxalate and calculus formation. Chronic irritation of the bladder epithelium secondary to crystalluria/bladder stones can then lead to neoplastic changes. Effects such as these on the urinary tract may be dependent on urinary pH and concentration, and in some cases this may explain interspecies and intersex differences in sensitivity, or modulation of toxicity by diet.

In a similar manner, factors affecting bile flow and composition may modulate primary and secondary hepatic pathology. Additionally, biliary metabolites, including endogenous bile acids, may cause changes in the colonic mucosa, either directly or following further metabolism by the gut microflora.

2.1.6.5 Sites of special sensitivity

The particular sensitivity of target organs for some xenobiotics commonly relates to the compound mimicking an endogenous physiological substrate or messenger in hormonally responsive tissues. A number of examples of such mechanisms and consequent target organs have been illustrated above. These include effects on the nervous system by cholinesterase inhibitors or overload of glutaminergic neurones, on the cardiovascular system by pressor amines and MAO inhibitors, effects on the thyroid by interference with the regulatory axis involving T4, T3 and TSH and effects on reproductive function by estrogens or anabolic steroids. In the last case, in addition to trophic or atrophic effects on the hormonally responsive tissues (e.g. endometrium, mammary tissue, testes), chronic exposure may lead to altered incidences in neoplastic changes in these tissues. In passing, it may be noted that the role of estrogens and progestogens in the oestrus cycle and pregnancy differs between rats and humans which needs to be borne in mind in interpreting effects on function of the reproductive organs.

Rapidly dividing tissues may represent another sensitive target site, particularly to cytotoxic agents acting at the level of nucleic acid or protein synthesis, and organs such as the gastrointestinal mucosa, bone marrow and hair follicle are common target sites for such substances.

The developing embryo is a particularly vulnerable target since abnormalities in organ growth and differentiation may result in irreversible life-time effects. In addition the period of organogenesis represents a short, but uniquely susceptible phase of the life cycle. This vulnerability is shown by the teratogenic effects of cytotoxic compounds discussed above and of vitamin A which is essential for normal foetal development, but

is teratogenic in overdose. The possibility of *in utero* effects is a focus for specific toxicity tests.

The spectrum of primary and secondary pathological change, and the target organs affected, together with their dose responsiveness can give a useful indication of the mechanisms of toxicity and facilitate the interspecies and dose-to-dose extrapolations in the risk assessment process.

2.2 Toxicokinetics

2.2.1 Introduction

The intensity of toxic action of foreign chemicals is normally directly related to the concentration of their active chemical entities at target sites. Thus, delivery to and presence of a toxicant at the target site is a determining factor for toxic outcomes. Toxicokinetics is a collective (mathematical) term which describes the disposition of foreign chemicals in the body by determining the types, rates and extent of absorption, distribution, metabolism and excretion of the chemical and is important for toxicological risk assessment. The term elimination includes both metabolic and excretory processes. The main routes of absorption, distribution and excretion of toxicants in the body are illustrated in Fig. 4a and 4b. Foreign chemicals generally enter the body by ingestion, inhalation or through dermal contact. They are absorbed into the blood circulation and distributed to the various organs and tissues. In general, foreign chemicals must be metabolically transformed, for a major part in the liver in order to be able to be eliminated from the body. The parent compound and/or its metabolites will be excreted either through the kidneys into the urine, via the bile and the gastrointestinal tract into the faeces, or if they are volatile liquids or gases through the lungs into the expired air. Other excretory pathways also exist, such as through saliva, sweat, and milk, which may be quantitatively important in certain instances (e.g. chloroorganic compounds in milk). Some poorly excreted chemicals may accumulate and be stored in body tissues such as fat and bone.

Toxicokinetic studies are performed to aid in the evaluation and interpretation of toxicological effects data (toxicological risk assessment). Mathematical models may be applied to describe parts or the whole process of disposition. In addition, toxicokinetic studies may provide data useful for dose level selection and timing in toxicodynamic studies. Furthermore, since the toxicokinetic behaviour of a chemical may vary between higher and lower doses, and between animals and humans, toxicokinetic information is very important when extrapolating effects data between these situations. Physiologically based toxicokinetic (PBTK) models which consist of several components where organs are grouped together according to blood flow are especially useful.

2.2.2 Absorption

To enter the body, a chemical must pass through many membrane barriers. These membranes have a high lipid content, thus chemicals which are lipophilic (fat soluble) cross body membranes by simple diffusion. Most organic molecules possess a certain

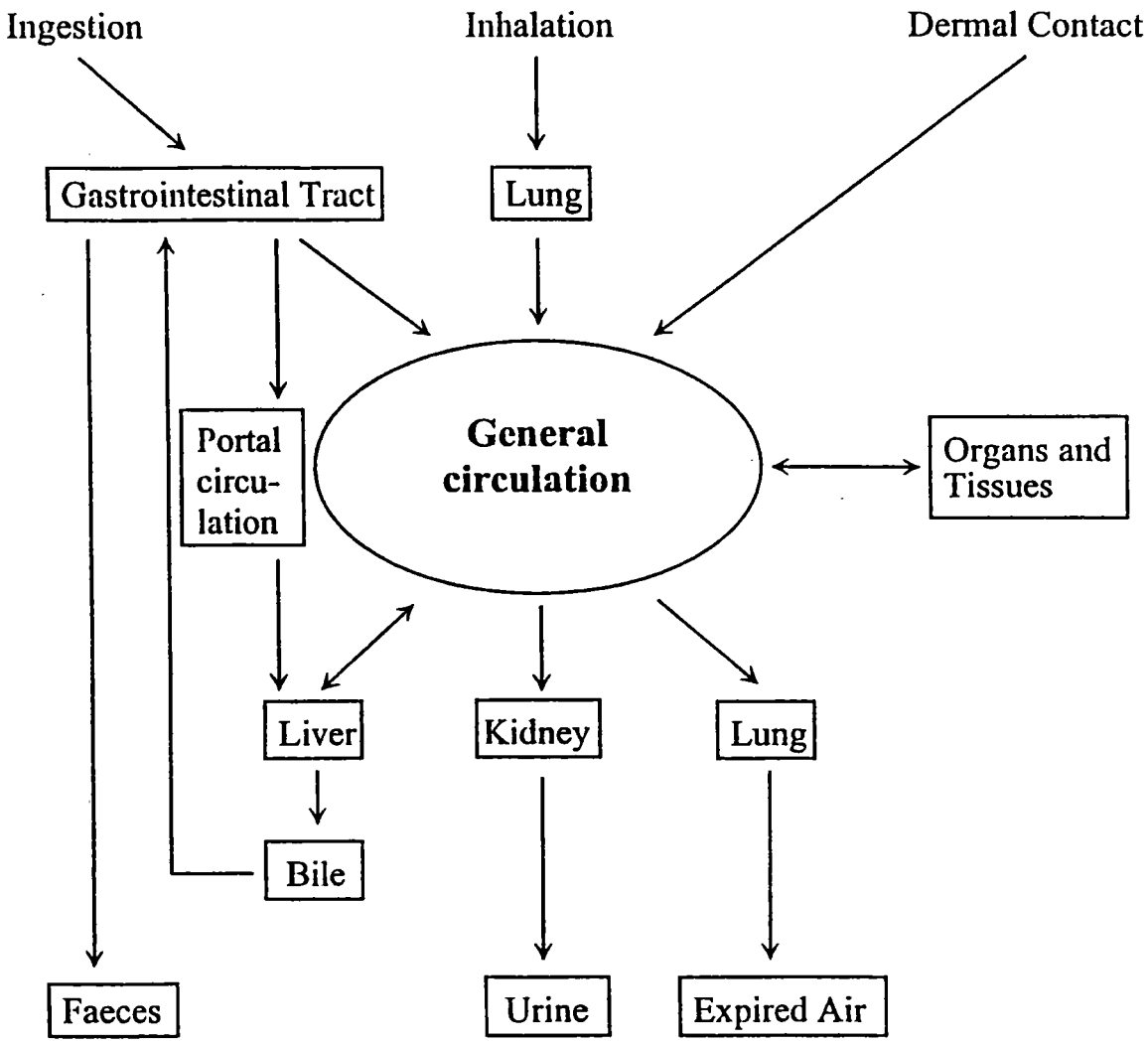

Fig. 4a. Main routes of absorption, distribution and excretion of toxicants in the body

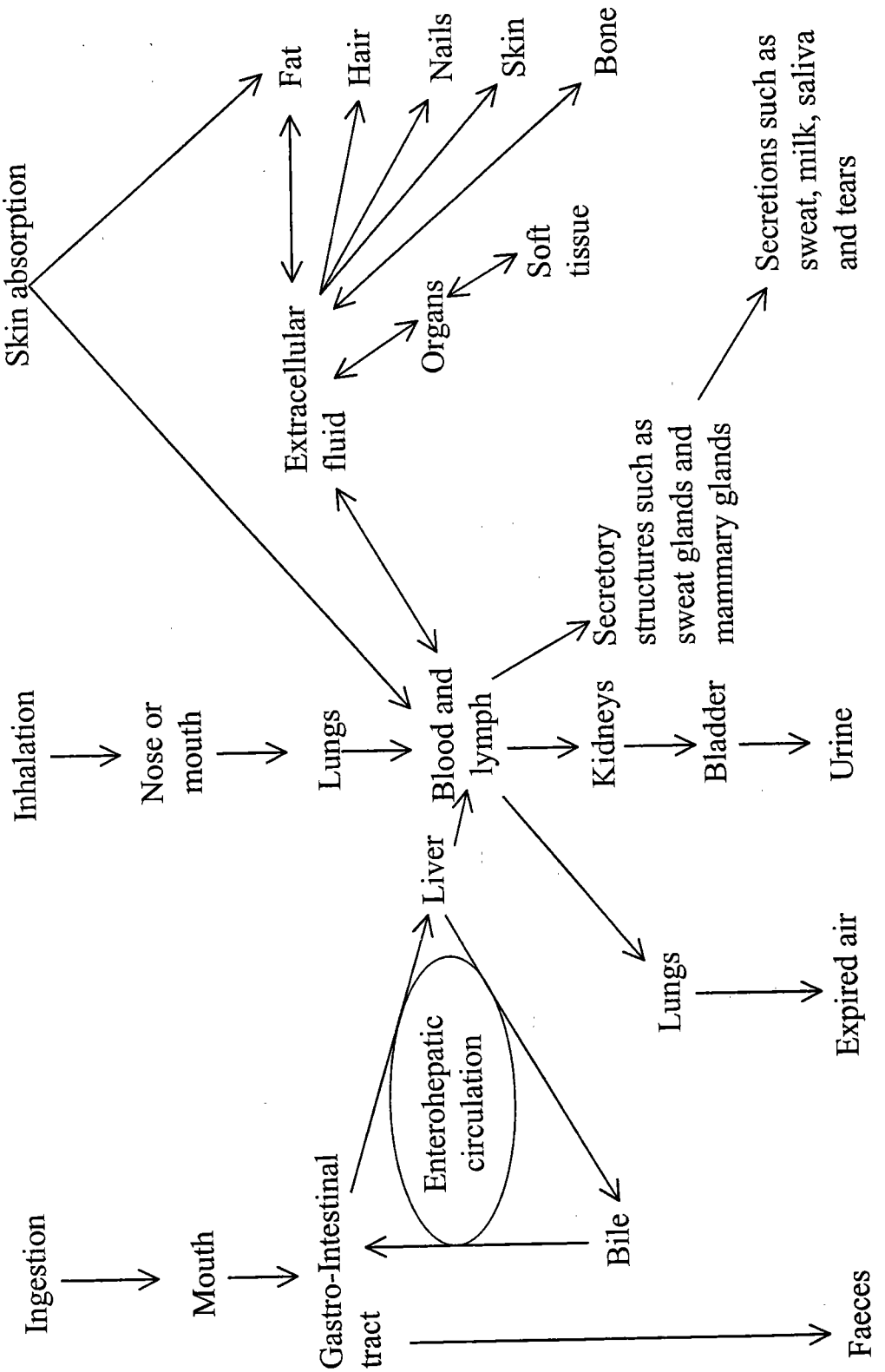

Fig. 4b. Main routes of absorption, distribution and excretion of toxicants

degree of lipophilicity and are thus absorbed more or less readily by diffusion. The rate of absorption of a chemical is directly related to its lipid/water partition coefficient and the concentration gradient across the membrane. Chemicals exist in solution in ionized and/or non-ionized forms. The charged (ionized) forms are generally much less able to penetrate cell membranes, this means that it is the non-ionized, lipid-soluble forms which are able to cross. For some chemicals which are not able to pass membranes by simple diffusion, there may be specialized transport systems. These systems may move chemicals against concentration gradients and require energy. Important transport systems for organic acids and bases exist in the central nervous system, the liver and the kidneys. Specialized transport systems exist in the gastrointestinal tract for some metals.

The gastrointestinal tract is one of the most important sites of absorption. Foreign chemicals may be present in the food supply or drinking-water and, after ingestion may be absorbed from the gastrointestinal tract. The extent and rate of absorption of a chemical depends on its physical properties, such as lipid solubility and dissolution rate. Absorption can take place along the full length of the gastrointestinal tract, including the mouth and rectum. The gastric juice is acidic, whereas the intestinal content is neutral to slightly basic. A chemical will be absorbed predominantly in the part of the gastrointestinal tract where it exists in its most lipid-soluble (non-ionic) form. Weak acids may therefore be absorbed in the stomach, whereas weak bases are preferentially absorbed in the intestine. Even if only a small percentage is present in the non-ionized, diffusible form, the very large surface area of the intestine, long contact time, high concentration gradients, as well as the rapid equilibration between ionized and unionized forms, combine to provide high absorption rates for many organic chemicals. The intestines contain a large number of different microorganisms (the intestinal microflora). The microflora may transform some ingested chemicals (e.g. reduction of azo compounds) so that different chemical moieties are absorbed than those ingested. Some chemicals are partly degraded by intestinal enzymes. The rate of gastrointestinal absorption of solid substances will depend on their dissolution rate, which in turn is dependent on size. Relatively large particles may therefore pass through the gastrointestinal tract without being absorbed. Many metals are also absorbed only to a limited degree from the intestine via specialized transport systems. There may be species differences in such systems, as well as in gastrointestinal physiology affecting absorption. Chemicals absorbed from the gastrointestinal tract (stomach, small intestine and most part of the large intestine) are carried by the hepatic portal vein to the liver where they are often metabolized before they reach the general circulation. This process is called first-pass metabolism, and can remove between 0 and 100% of the dose depending on the chemical and animal species. In addition first-pass metabolism may be dose-dependent giving a disproportionate increase in internal dose with increase in dose administered.

Chemicals absorbed by inhalation are either gases, vapours of volatile liquids or aerosols (particles, mists, fogs). Absorption of inhaled gases (and vapours) takes place mainly in the lungs. If the gas is very water soluble, it may be dissolved in the mucus covering the cells of the upper airways. Very reactive and hydrophilic gases may also react with cell surface components of the upper airways and thus not always reach the lower airways and alveoli. In the lungs, equilibrium is usually established very rapidly

between the concentration of the gases in the alveolar air and the circulating blood in the lung capillaries. Theoretically, the concentration in the blood of an absorbed gas is dependent on its solubility in blood, the more soluble a chemical is, the longer time it takes to reach an equilibrium with body water. Increasing the respiration rate does not change the rate of absorption of a very soluble gas, whereas increasing cardiac output markedly enhances this parameter. However, in practice the respiration rate and heart rate usually increase in concert. During repeated inhalation exposure, a steady state (equilibrium) is reached between the concentration of the chemical in the air and that in the body.

Aerosols such as particles, fibres (e.g. asbestos and man-made mineral fibres) and liquid droplets, are deposited along the respiratory tract and in the lungs dependent on their size. Aerosols of 5 μm or larger are usually deposited in the nose and throat. Aerosols of 2 to 5 μm are mainly deposited in the lower respiratory tract (trachea, bronchi, and bronchioles), whereas aerosols of 2 μm and smaller gain access to the alveoli. Aerosols less than 1 μm may leave the alveolar space by exhalation. In addition, the deposition of aerosols such as fibres, depends on their geometrical shape. Deposited particles are cleared by retrograde movement of the mucus layer in the ciliated portions of the respiratory tract (so-called muco-ciliary clearance). Particles cleared in this way are swallowed and may be absorbed from the gastrointestinal tract. Aerosols in the alveoli may be absorbed into the blood or cleared by alveolar scavenging cells (macrophages). Absorption of aerosols is also dependent on their solubility characteristics. Some particles may be carriers of other chemicals, and thus present these for absorption in various regions of the respiratory tract and the lungs.

The skin is a relatively good barrier to chemicals, however, many organic chemicals can be absorbed through the skin in sufficient quantities to produce general toxic effects. Absorption of chemicals through the skin is variable being influenced by skin area, nature of exposure, e.g., vapour deposition compared to direct liquid contact, formulation, work practices, air temperature and weather conditions may also influence potential exposure. A chemical must cross a large number of cell layers of the skin before it can reach the circulation, the rate determining layers is the outermost, horny layer of the outer epidermis. All toxicants appear to move across the horny layer by passive diffusion. Absorption through glands and hair follicles seems to be of much less importance. Permeability of the skin for polar substances depends on the lipophilicity of the chemical, its molecular weight and on the diffusivity and the thickness of the horny layer. Polar substances can diffuse through the protein filaments. These different aspects vary both with the site within a species and also generally between species. In general, the skin of rats and rabbits appears to be more permeable than that of humans, whereas the skin of guinea-pigs, pigs and monkeys is often similar in permeability to humans. Damage to the skin (e.g. abrasion, corrosion, inflammatory disease) may markedly increase its permeability. Co-exposure to some solvents (e.g. acetonitrile, dimethyl sulfoxide) may also increase dermal absorption. Sweat formation under impermeable covers, such as protective gloves, can hydrate the horny layer thereby increasing its permeability. It can be assumed that a compound that is well absorbed through the skin will also be well absorbed through the gastrointestinal tract.

2.2.3 Distribution

When a chemical enters the systemic circulation after absorption, it is distributed (translocated) to the various tissues and organs in the body. Chemicals absorbed from the greatest part of the gastrointestinal tract are transported by the portal circulation to the liver before they reach the general circulation. Such chemicals will often be metabolized during passage through the liver, thereby affecting their distribution and toxicity (first pass metabolism). The rate of tissue distribution is primarily determined by the organ blood flow and the rate of diffusion through the capillary bed and into the cells. The extent of distribution is also affected by the affinity of the chemical for binding sites in the blood and the tissues. The penetration of chemicals into cells occurs mostly by passive diffusion of their lipophilic moieties. Small water-soluble molecules and ions may gain access through aqueous pores, whereas larger, water-soluble molecules and ions only can enter by special transport systems. Some sites of the body are less penetrable by chemicals than others because of the presence of tight junctions between adjacent cells. This is particularly true for the central nervous system (the so-called blood-brain barrier). This is not an absolute barrier, but it is much more restrictive to passage of water-soluble molecules and ions than other parts of the body. The blood/thymic barrier is also rather restrictive. Other less effective barriers include those between the blood and the conceptus (the placental barrier) and between the blood and the testis. In certain instances, chemicals may be concentrated in the fetus due to specific mechanisms. There are considerable developmental and species differences in the relative efficiencies of these barriers.

Many chemicals may bind reversibly or dissolve in body constituents, thereby impeding their even distribution throughout the body. Some chemicals may have a high affinity to their target site (e.g. carbon monoxide to haemoproteins such as haemoglobin), other chemicals may concentrate in tissues without appreciable effects in that tissue (e.g. DDT in fat, lead in bone). Albumin in blood plasma is known to function as a binding depot and transport protein for a number of chemicals, other more specific transport proteins also exist in the plasma. The liver and kidney have a high capacity to bind many different chemicals, for example a special binding protein exist for cadmium and zinc. The tissue where a chemical is concentrated can be looked upon as a storage depot. The concentration of a chemical in a storage depot is always in equilibrium with its free (unbound) fraction. As a chemical is eliminated, more is released from the stored form. High binding and storage of chemicals will markedly affect their distribution in the body and slow their elimination.

2.2.4 Metabolism

Lipophilic substances entering the body would accumulate and remain there for a long time were it not for systems being able to convert most of such substances to hydrophilic (water-soluble) compounds. Metabolism (biotransformation) refers to the processes by which foreign chemicals are structurally altered by enzymatic reactions.

A parent molecule may be modified at a number of positions and the various metabolites may be further modified enzymatically. Many foreign chemicals undergo a

two-step, sequential biotransformation (Fig. 5), whereby in the first step (Phase I) they are usually oxidized to form polar (less fat soluble, more water soluble), primary metabolites. These may be further metabolized via a second step (Phase II) by conjugation (coupling) reactions. The conjugates are often organic acids which are very hydrophilic and thereby readily excreted via the kidneys and/or via the bile. In Phase I metabolism, functional groups are introduced into the molecule which increase the polarity or provide a "handle" for subsequent conjugation with highly polar endogenous substrates such as glucuronic acid or sulfate. Chemicals which show intermediate polarity may only be conjugated via phase II before they are excreted.

The liver has generally the highest capacity for metabolism of many types of foreign chemicals and most of the enzymatic reactions which convert lipophilic substances to hydrophilic conjugates may be found in this organ. However, foreign compound metabolism may occur in most tissues of the body, with lung, kidney and intestines usually having intermediate capacities and skin, gonads, placenta and adrenals having fairly low capacities. In many instances, extrahepatic biotransformation is important for determining organ-specific toxicity such as in the kidneys, lungs and gonads. These organs are composed of many different cell types, some of which may have high specific activities of some metabolic enzymes, even though their activities with respect to the whole organ may be fairly low.

Phase I reactions

Foreign chemicals may be toxic as such, phase I-metabolism will mostly decrease their toxicity (detoxication or inactivation). However, in certain instances will the metabolite be more toxic than the parent compound after Phase I-metabolism (toxication or metabolic activation). Oxidation is the most important Phase I metabolic pathway, it may be looked upon as the addition of a hydroxyl moiety to a foreign chemical. Some chemicals may also undergo reduction or enzymatic hydrolysis during phase I-metabolism. The most important oxidation enzymes are the cytochrome P-450 monooxygenases (P-450), haeme proteins that play critical roles in the bioactivation and detoxification of a wide variety of xenobiotic substances. These enzymes exist in a number of different forms (up to 30 per mammalian species have been identified) belonging to 13 distinct gene families in mammals. These enzymes differ in the reactions they catalyse. The content and activities of the different isoenzymes may vary dependent on organ, species, age, health, sex and they may be altered by exposure to various chemical substances. In humans, certain segments of the population may show differences in their contents of individual P-450 forms, thereby metabolizing foreign chemicals differently than the rest of the population (polymorphisms); such groups may show increased or decreased effects to substrates for the specific form of P-450 involved.

Currently more than 700 P-450s have been characterized, inclusive of the many different species of organisms that have been studied. Based primarily on sequence similarities, a standardized nomenclature has been adopted that categorizes the individual P-450s into respective families and subfamilies. P-450 proteins exhibiting > 40% similarity in protein sequence are classified within the same family, while proteins exhibiting > 55% sequence similarly are grouped in the same sub-family.

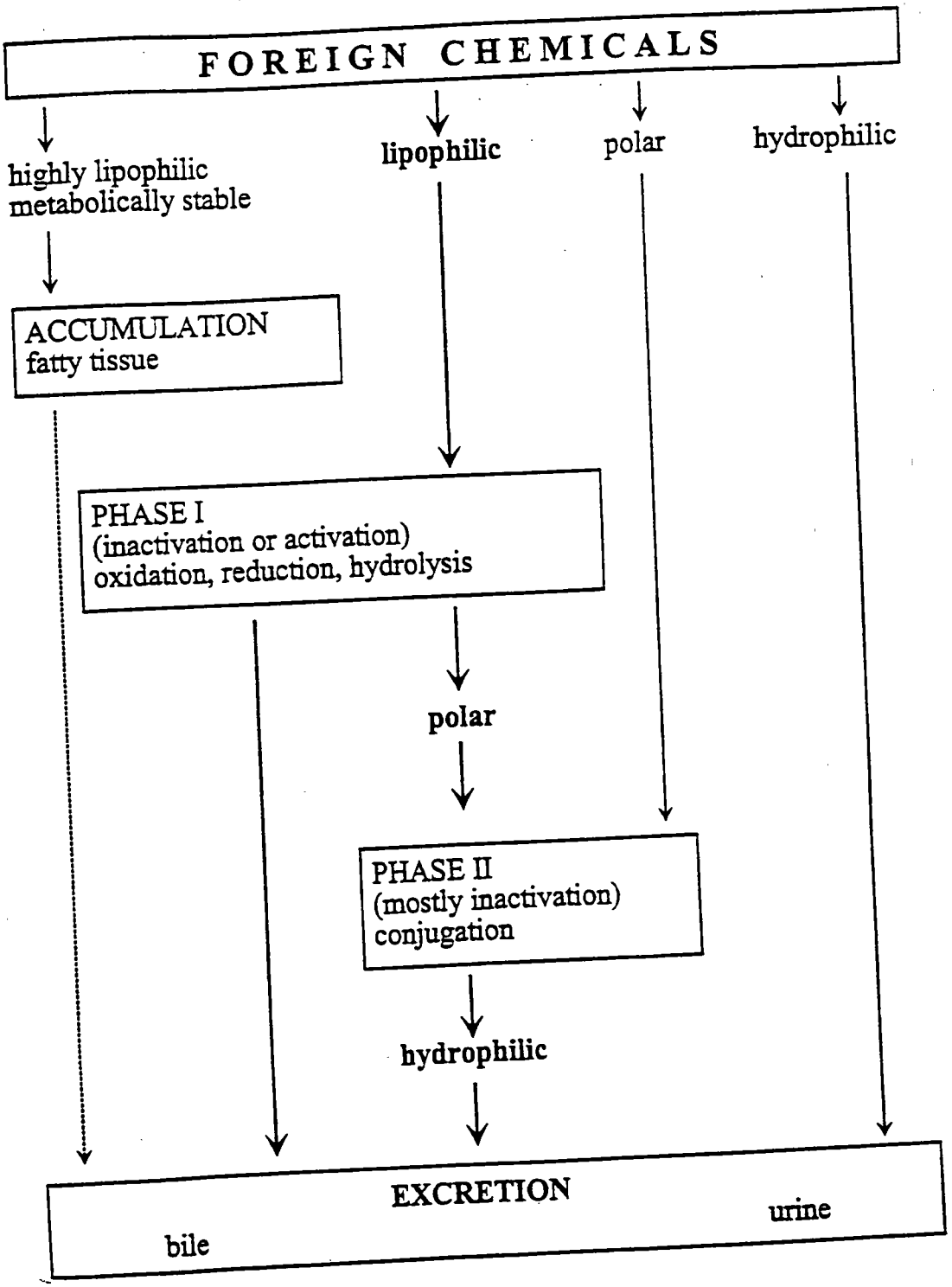

Fig. 5. Metabolism of foreign chemicals in the body

The CYP1 family includes two sub-families, CYP1A and CYP1B. The CYP1A family members have been well characterized and historically linked to the aryl hydrocarbon receptor as inducible genes. The CYP1A enzymes activate benzo(a) pyrene and other polyaromatic hydrocarbons (for CYP1A1) and aromatic amines such as 2-acetylaminofluorene, heterocyclic amines and aflatoxin B1 (for CYP1A2). CYP1A1 is expressed in many tissues but typically only after induction with TCDD or other Ah receptor ligands. In contrast, CYP1A2 is primarily expressed in the liver at significant constitutive levels and is highly inducible in this tissue via the Ah receptor pathway.

Many of the P-450 genes are known to exist in variant forms or polymorphisms that have differing activities. One of these, CYP1A1 catalyses the oxygenation of PAHs such as benzo(a)pyrene producing certain metabolites that are highly reactive with DNA. CYP1A1 is induced by cigarette smoke, dioxins and PAHs. Individuals vary widely in the extent to which CYP1A1 is induced by such exposures, with 10% of the Caucasian population being highly inducible. The level of inducibility varies by 20-fold in the human liver while the enzyme differs by more than 50-fold in human lung tissues.

Genetic variation in other cytochrome P-450s can also be important modulators of cancer risk. CYP1A2 metabolizes arylamines, heterocyclic amines and aflatoxins. In human liver there is a greater than 40 fold variation in expression of the CYP1A2 gene. CYP2E1 metabolizes *N*-nitrosamines, butadiene, benzene and carbon tetrachloride. The activity of CYP2E1 in humans varies by 50-fold.

The P-450 enzymes show great versatility with respect to the reactions in which they participate; in all 60 different chemical reactions have been identified. Examples of the types of oxidations which P-450 enzymes undertake are: aliphatic hydroxylation, aromatic hydroxylation, epoxidation, *N*-, *O*- or *S*-dealkylation, deamination, *N*-hydroxylation, sulfoxidation, desulfuration and oxidative dehalogenation. Other oxidation reactions may be catalysed by the flavin-containing monooxygenase or peroxidases. An important hydrolytic enzyme is the epoxide hydrolase which converts potentially toxic epoxides to less toxic dihydrodiols. Other Phase I enzymes may hydrolyse esters and amides or oxidize alcohols and aldehydes.

Phase II reactions

In contrast to the phase I P-450 activating enzymes, phase II enzymes (epoxide hydrolase, glutathione *S*-transferase, *N*-acetyl transferases and sulfotransferases) generally detoxify carcinogenic metabolites by conjugating them with glucuronide, glutathione or sulfate to produce hydrophilic products that are readily excreted.

Phase II metabolism may be considered (in nearly all cases) as inactivation or detoxication reactions. The Phase II conjugation reactions are biosynthetic and thus require energy. One of the major Phase II reactions is glucuronidation whereby a molecule of glucuronic acid is coupled to the substrate via an enzyme system called UDP-glucuronosyltransferase. This system consists of two multi-gene superfamilies. Subfamily 1 (UGT1) consists of at least four enzymatic forms catalysing the glucuronidation of phenols and bilirubin, whereas steroids and bile acids are

glucuronidated by forms belonging to the UGT2 subfamily. Glucuronides below a molecular weight of 250 are preferentially excreted by the kidneys, whereas glucuronides above a molecular weight of 350 are mostly excreted in the bile of rats; the molecular weight threshold for humans is about 500. The other major conjugation reaction is sulfation, whereby Phase I-metabolites containing a hydroxyl group (phenols, aliphatic alcohols) are coupled with inorganic sulfate through sulfotransferases. In humans, there are two enzymatic forms of phenol sulfotransferase and one hydroxysteroid sulfotransferase. Sulfate conjugates are excreted mainly in the urine. In general, glucuronidation has a higher capacity than sulfation which may be saturated at high doses due to depletion of the activated sulfate co-factor. The major pathway of conjugation can be influenced by dose, with sulfation being more important at lower doses.

Conjugation of chemically reactive compounds (electrophiles) with the tripeptide glutathione directly or via the glutathione *S*-transferase (GSTs) enzymes is an important detoxication pathway in the body. The cytosolic glutathione *S*-transferases consist of four multi-gene families (alpha, mu, pi, theta) and are widely distributed in organs and tissues. Multiple forms of the enzyme provide broad overlapping specificities for chemicals being catalysed. The glutathione conjugates may be excreted in the bile or converted by several further enzymatic steps to mercapturic acids and excreted in the urine. An important biotransformation route for substances containing an amine function is acetylation via *N*-acetyl transferases. They are coded by two distinct genes, one of which is polymorphically distributed in humans. Other conjugation reactions known to occur are methylation and amino acid conjugation.

The balance between phase I and II enzymes determines the molecular dose of carcinogens, thereby substantially influencing cancer risk. For example, glutathione *S*-transferase M1 (GSTM1) detoxifies a number of reactive, electrophylic substances, including the carcinogenic PAHs, ethylene oxide and styrene. Approximately 50% of Caucasians have a deletion in the GSTM1 gene. The null GSTM1 genotype or its phenotypic expression has been quite consistently (although not uniformly) associated with increased risk of bladder and lung cancers. Fig. 7 illustrates an example of an important solvent and environmental contaminant, perchloroethylene (PER) that is known to be metabolized by both cytochrome P-450 and glutathione-dependant biotransformation pathways resulting in formation of reactive metabolites which may covalently bind to cellular macromolecules.

2.2.4.1 Modulation of metabolism

A number of factors may affect the rates of metabolism of foreign chemicals. These factors include species, strain, age, sex, time of day, nutrition and disease states. In addition, the amounts and activities of the enzymes may be increased following treatment with chemicals (enzyme induction) or they may be inhibited by the presence of another chemical or by other means, such as depletion of co-factors. Many different chemicals have been shown to cause enzyme induction with variable effects on the different types of reactions and enzyme systems involved. Many chemicals induce the P-450 enzymes, but the onset, magnitude and duration of induction, as well as the

induction profile of individual P-450 isoforms, may vary. The outcome of enzyme induction may often result in an increased detoxication and increased rate of excretion of other chemicals, but in some instances induction may increase a metabolic activation pathway and thus enhance toxicity. Induction of specific P-450 forms may make certain individuals particularly sensitive to chemicals which are activated by the induced isoenzyme. The most widely studied enzyme inducers are phenobarbital, polycyclic aromatic hydrocarbons and polychlorinated compounds. The latter two types seem to operate through a common mechanism by binding to a specific cellular site, the Ah (aromatic hydrocarbon) receptor. The inducer-receptor complex binds to DNA in the nucleus and there turns on gene expression leading to higher P-450 synthesis.

2.2.4.2 Metabolic activation

Most metabolic reactions lead to the formation of less toxic products. However, in certain instances enzymatic catalysis generates reactive, electrophilic intermediates (Fig. 6). If such reactive intermediates are not rapidly detoxified they may undergo covalent (irreversible) linkage with tissue macromolecules. The covalent reactions with DNA have been extensively studied as they may be linked to the first stage of chemical carcinogenesis (see Fig. 6). If this macromolecular binding is not repaired, it may result in various types of toxicity. The formation of reactive intermediates is known to be involved in acute tissue damage and cell death (necrosis), genotoxicity and cancer initiation, as well as in generating haptens involved in antigen-antibody reactions (Table 4). Many of the metabolic activation reactions are due to the involvement of P-450 enzymes, however, many of the other metabolizing enzyme systems may produce reactive electrophiles, even conjugating enzymes such as sulfotransferases, *N*-acetyl transferases and glutathione *S*-transferases. In the case of glutathione *S*-transferases the reactive product may be formed after direct conjugation with glutathione to form a reactive episulfonium ion, or after multiple enzymatic processing of glutathione conjugates to form reactive thiols via the C-S lyase pathway. For chemicals which are metabolically activated, the toxic outcome will depend on the balance between rates of activation and detoxication. There are many conditions that can disturb this balance and thus affect toxicity. Enzyme induction can increase the overall rate of metabolism, which in turn can lead to an excess production of a reactive intermediate. Large doses of a foreign chemical can rapidly deplete cellular defense mechanisms. At high doses, a shift from a detoxication pathway to an activation pathway may also occur.

2.2.4.3 Species and individual differences in metabolism

There may be both qualitative and quantitative species differences in metabolism of foreign chemicals. Qualitative differences may be due to defective enzymes in certain species or to unique species reactions. Quantitative variations may be due to differences in enzyme concentrations, differences in the profile of enzymatic forms or in the extent of competing reactions. Most of the species differences are of a quantitative nature, and many are due to differences in the relative amounts and activities of P-450 enzymes. Species-specific defects in enzymatic reactions may either lead to an unique sensitivity towards a foreign chemical (defective enzymatic detoxification reaction) or confer resistance to it (defective enzymatic activation reaction). Dogs are for example unable to

Table 4. Examples of toxicities caused by metabolic activation

Compound	Metabolic pathway	Toxicity
2-Acetylaminofluorene	*N*-Hydroxylation and subsequent reactions	Liver necrosis and cancer
Aflatoxin B_1	Epoxidation	Liver necrosis and cancer
Benzene	Epoxidation and subsequent reactions	Bone marrow toxicity and leukaemia
Benzo(a)pyrene	Epoxidation and subsequent reactions	Lung cancer
Carbon tetrachloride	Free radical formation	Liver necrosis
Chloroform	Oxidation to form phosgene	Liver and kidney necrosis
1,2-Dibromo-3-chloro-propane	Glutathione conjugation	Testis and kidney necrosis
Dimethnylnitrosamine	α-Hydroxylation and subsequent rearrangement	Liver necrosis and cancer
4-Ipomeanol	Epoxidation	Liver, lung and kidney necrosis
Parathion	Oxidation with release of sulfur	Inhibition of nervous tissue cholinesterase
Vinyl chloride	Epoxidation	Liver cancer

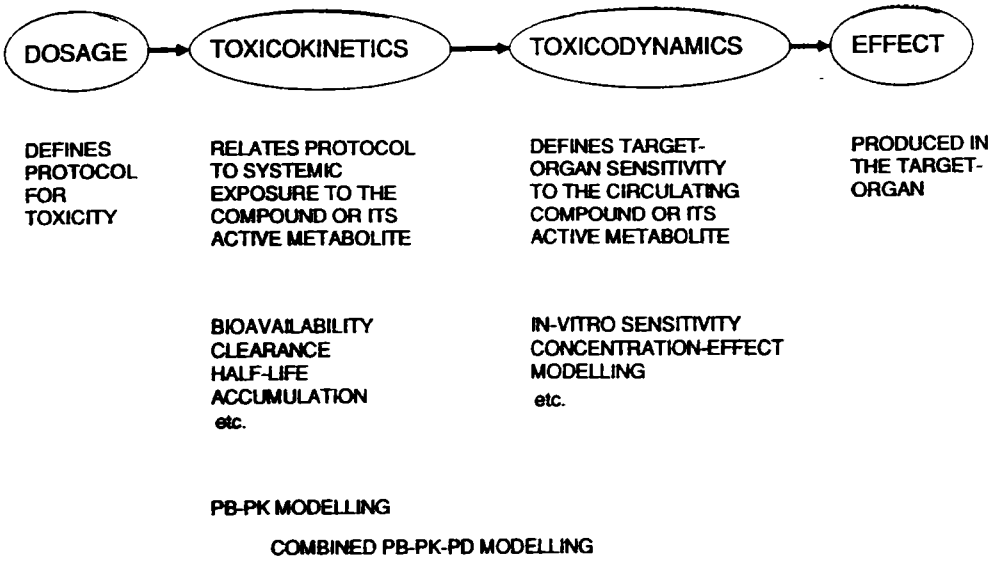

Fig. 6. Role of metabolism in chemical-induced toxicity

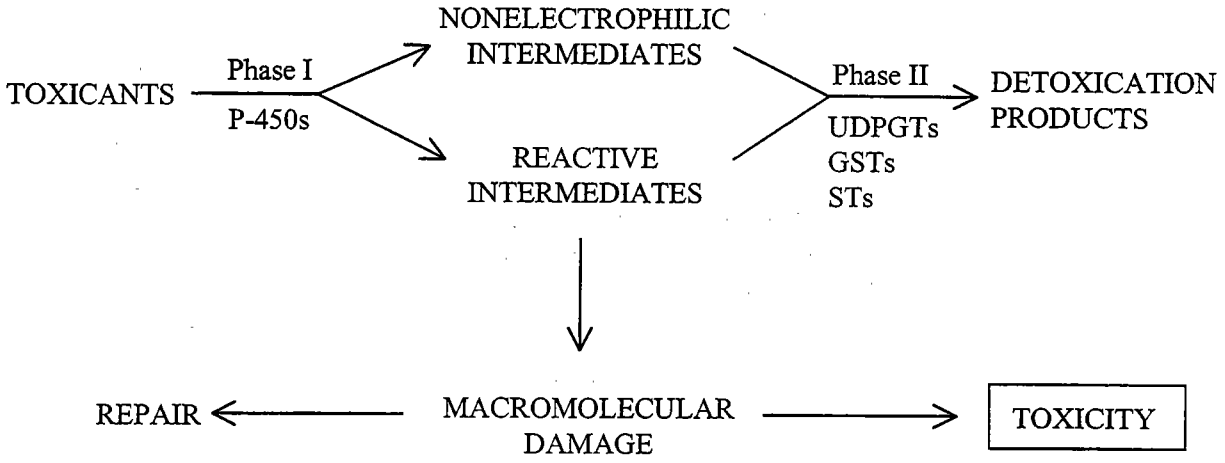

Fig. 7. Toxicokinetics and toxicodynamics

acetylate aromatic amino compounds, whereas cats are defective in glucuronidation and pigs in sulfation (Table 5). There are also species differences in the composition and distribution of the bacterial flora within the gastrointestinal tract, which may affect the metabolism of some compounds. There may also be animal strain differences in metabolism.

Genetically similar animals are usually used in toxicological studies to avoid the added complexity of interindividual differences in metabolism and response. There is much more interindividual variation in the metabolism of foreign compounds in humans compared to laboratory animals, some of which may have a genetic basis. Such differences may be of major importance for the interindividual differences in susceptibility towards foreign chemicals. Examples of quantitative differences in rates of carcinogen metabolism with human liver preparations are: 44-fold for 4-aminobiphenyl, 30-fold for 2-naphthylamine, 15-fold for dimethylnitrosamine, 11-fold for benzo(a)pyrene and 5-fold for 2-acetylaminofluorene. Genetically determined polymorphisms in humans are known to occur, both in Phase I and Phase II metabolic reactions (Table 6). For instance, the population is bimodally distributed with respect to one of the *N*-acetyltransferases, whereby some individuals are able to metabolize substrates, such as aromatic amines rapidly (rapid acetylators), whereas other individuals metabolize such compounds more slowly (slow acetylators). Among Caucasians, slightly more are slow acetylators compared to rapid acetylators, whereas among Chinese and Japanese populations rapid acetylators predominate. Slow and fast acetylators show differences in the magnitude of therapeutic response and in the propensity towards side effects.

Table 5. Variability in xenobiotic metabolism

a) **Genetic - interspecies**

- extent of metabolism by different pathways shows wide variability
- some species are deficient in certain reactions especially conjugations, e.g.,

cat	-	glucuronidation
dog	-	*N*-acetylation of $ArNH_2$
pig	-	sulfation
Indian fruit bat	-	glycine conjugation

b) **Genetic - intraspecies**

- inbred strains of rats and mice may show wide variations in metabolic capacity
- heterogeneous populations may show 2 or more discrete subpopulations (genetic polymorphisms), e.g.,

C-oxidations	-	humans (debrisoquine)
hydrolyses	-	humans (succinylcholine)
acetylation	-	humans and rabbits

c) **Environmental**

- sex steroids (especially in rat)
- nutrition
- enzyme inducers

dietary	-	indole derivatives
	-	polycyclic aromatics
environmental	-	chlorinated hydrocarbons
	-	terpenes
	-	smoking (polycyclic aromatics)
pharmaceutical	-	phenobarbitone, rifampicin

- enzyme inhibitors

dietary	-	bioflavonoids
pharmaceutical	-	cimetidine

- competing substrates - especially if capacity is limited, e.g., sulfation
- age - lower enzyme activity in neonatal and geriatric groups
- disease - can affect metabolism either directly via enzyme activity or indirectly via changes in excretory capability

Table 6. Examples of functional metabolic polymorphisms

Enzymatic forms	Typical substrates
Cytochrome P-4501A1	Polycyclic aromatic hydrocarbons
Cytochrome P-4501A2	Aryl and heterocyclic amines
Cytochrome P-4502A6	Coumarin
Cytochrome 2C19	Drugs (mephenytoin, diazepam, omeprazol)
Cytochrome 2D6	Over 30 clinically used drugs
Cytochrome 2E1	Ethanol, organic solvents, nitrosamines, paracetamol
Cytochrome 3A5	Various drugs
N-Acetyl transferase NAT2	Aryl amines
Sulfotransferase TS-PST	Aryl and heterocyclic amines
Glutathione *S*-transferase M1	Polycyclic aromatic hydrocarbons, oxides and diol-epoxides

2.2.5 *Excretion*

In urinary excretion, foreign chemicals in the circulation are filtered at high rates in the glomeruli of the kidneys and appear within the lumen of the kidney tubules. If the chemical is lipophilic, it is rapidly reabsorbed across the tubular cells back into the bloodstream. Thus, lipophilic substances will remain for prolonged periods in the body. On the other hand, water-soluble substances such as hydrophilic metabolites are not reabsorbed, but excreted from the tubules into the urine. Metabolism and urinary excretion are thus tightly coupled for the efficient elimination of foreign chemicals from the body. Organic anions and cations may in addition to glomerular filtration be actively secreted into the urine by tubular transport systems. The urinary excretion of weak acids may be increased by alkalinization of the urine, because this will increase the percentage of ionization of the acid.

Biliary excretion is another major pathway for excretion of foreign chemicals from the body. The detection of foreign chemicals in the faeces may either be due to their passing through the gastrointestinal tract unabsorbed, or due to excretion via the bile. In addition, some chemicals are translocated directly across intestinal cells from the blood and into the intestinal lumen. Chemicals absorbed in the gut are directly transferred to the liver via the portal circulation. The liver can extract such chemicals and metabolize them, thereby avoiding exposure of the parent compound to other parts of the body (first pass metabolism). The metabolites may thereafter be directly excreted into the bile or into the blood. Conjugated metabolites appearing in bile are often actively transported from the liver cells. After the metabolite is excreted into the bile, it appears in the small intestine, where it either can be reabsorbed (so-called enterohepatic circulation) or eliminated with faeces. Increases in biliary flow induced by chemicals may increase the biliary excretion of other chemicals and thus decrease their toxicity.

Chemicals which are gaseous at body temperature are primarily excreted by the lungs. Volatile liquids may also be excreted by exhalation in amounts in proportion to their volatility in water. Such chemicals are excreted by simple diffusion, gases and volatile liquids with high solubility in blood are therefore excreted slowly compared to chemicals with low solubility. Elimination of gases via the lungs is approximately inversely proportional to their rate of absorption. When a steady state is reached during continuous exposure, there is a constant rate of elimination of a gas or a vapour in the exhaled air.

2.2.6 *Kinetic modelling*

The time course of the disposition (absorption, distribution, metabolism, excretion) of a foreign chemical may be modelled and described in mathematical terms. Each of the basic kinetic processes may be characterized by parameters which specify the extent to which the process occurs and the rate at which it occurs. Such parameters are often calculated from data on blood plasma concentrations.

Toxicokinetic studies and kinetic modelling constitute important parts of the safety evaluation of chemicals. Such information allows a comparison of the amount and rates of absorption, distribution, metabolism and elimination (metabolism and excretion) with the types, extent and severity of observed toxic effects. In cases where the toxicity of a chemical is due to a specific metabolite, toxicokinetic data will be able to define the extent of absorption of the parent chemical, its delivery to the site of metabolism, the extent of formation of the metabolite, and its delivery to the target organ. Additional important data include the influence of dose on the fate of the chemical and the extent of accumulation of the parent compound and/or active metabolites on repeated exposure to the compound. Kinetic modelling is a very useful tool for describing route-, dose- and species-dependent differences in chemical disposition. However, a great deal of information is required to perform this modelling, and attention must be paid to the range of human diversity in many parameters related to toxicokinetics, including rates of metabolism of specific pathways. Fig. 8 illustrates the paradigm of toxicokinetics and toxicodynamics.

A number of useful parameters and constants may be derived from toxicokinetic data. The determination of the area under the plasma concentration-time curve (AUC) after oral administration compared to the AUC after the same dose given intravenously, will determine the fraction of the dose reaching the systemic circulation unchanged (bioavailability). Two compounds may show similar bioavailability, but may be absorbed at different rates. The extent of distribution is characterized by the apparent volume of distribution. This is the theoretical volume in which an absorbed substance appears to be evenly dissolved (i.e. total body load divided by the plasma concentration). If the binding of the substance to plasma proteins is high, little of the chemical may exist outside the circulation giving a small volume of distribution. If on the other hand plasma binding is low but tissue binding is high, the chemical will have a large volume of distribution.

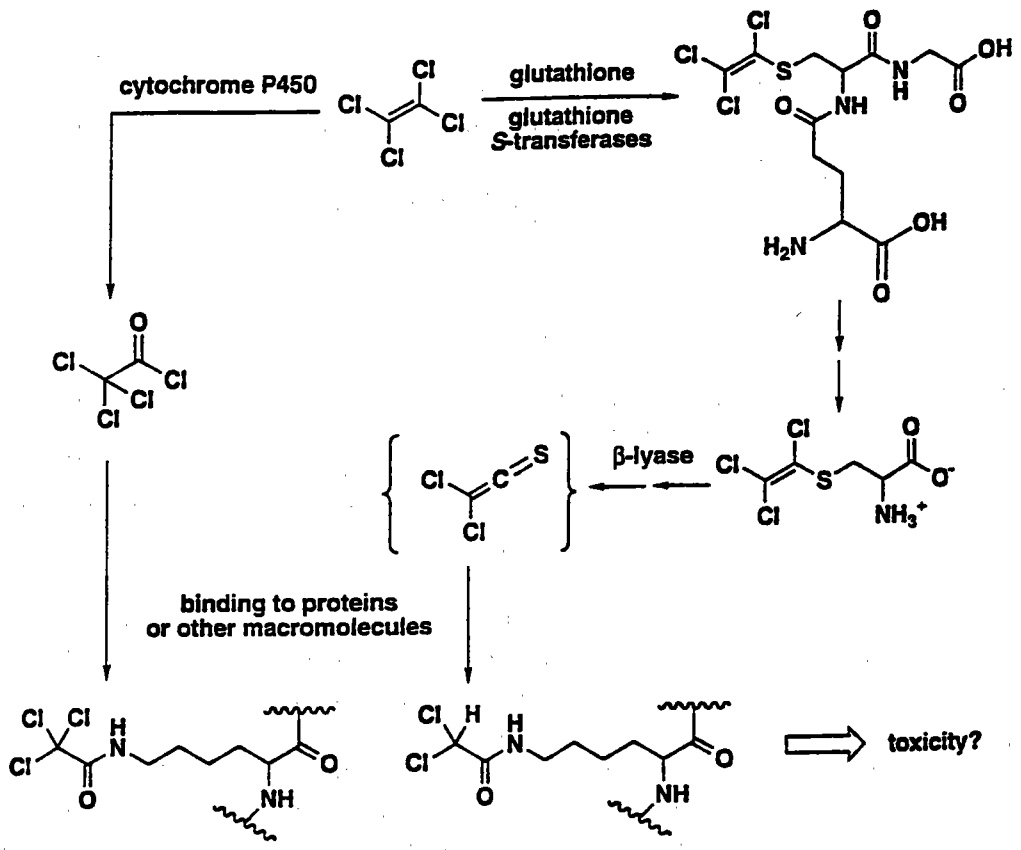

Fig. 8 Protein adduct formation in Per-metabolism

Clearance is another important parameter which relates a substance's rate of transfer or elimination to its concentration in plasma. The overall efficiency of removal of a chemical can be characterized by clearance. Clearance may mathematically be derived by the dose that is available to the organs of elimination divided by the AUC. High clearance values indicate efficient and rapid elimination, whereas low values indicate slow and less efficient removal of the chemical from the body. Elimination reactions are usually exponential and proceed at a rate which is proportional to the concentration of the chemical (first-order processes). The time taken for the concentration to fall to 50% of the initial value is constant and independent of concentration. This parameter is the half-life of the elimination process. Rapidly eliminated compounds have short half-lives, whereas slowly eliminated compounds have long half-lives. The half-life may be calculated by the volume of distribution multiplied by 0.693 (the natural logarithm of 2) divided by the clearance. It should be recognized that the half-life is usually based on plasma concentrations of the chemical and that plasma measurements may not be sufficiently sensitive to reflect the sequestration and slow release of a chemical from minor site even if this is the site of toxicity, for example the prolonged retention of canthaxanthin in the eye.

In the past, classical toxicokinetic modelling has been used to represent the body as consisting of one or a small number of theoretical, non-physiological compartments. More recently, physiological based kinetic models have been developed where mass balance equations allow modelling of each tissue or organ. Both classical and physiological kinetic (PB-PK) models are very useful for describing the fate of a foreign chemical in the body, whereas only the physiological kinetic models are able to predict tissue concentrations. In physiological kinetic modelling the body is envisaged as being comprised of a small number of physiologically relevant compartments. The models are characterized by physiological parameters such as tissue volumes and blood flow rates, biochemical parameters such as partition coefficients, and kinetic parameters for metabolism and removal. Physiological kinetic models have been used to describe route-dependent, dose-dependent and species-dependent differences in toxicokinetics. However, the parameters used in physiological kinetic modelling are often unknown or known with imprecision which may limit the utility of such models. On the other hand, physiological models have, in many instances, been very valuable in extrapolating and interpreting animal data in relation to possible human exposure.

During repeated exposure to a foreign chemical, the chemical will accumulate in the plasma and tissues if the half-life is about the same or greater than the exposure interval. In this situation, accumulation will proceed until an equilibrium or steady state is reached when the rate of elimination equals the rate of input. For compounds which are eliminated monoexponentially, it will take continued exposure for 4–5 times the elimination half-life to reach steady state. Compounds with very long half-lives will accumulate appreciably during chronic intake. Some foreign chemicals, especially lipophilic chemicals that are stored in adipose tissue, have extremely long half-lives due to very limited metabolic transformation. One such example is TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) with an estimated elimination half-life of 7–9 years in humans. This means that it would take about 30–45 years of exposure to reach steady state during constant intake.

Many toxicokinetic processes have finite capacities, these may become saturated by the presence of excess chemical. Both metabolic enzymes, transport processes and plasma protein binding can become saturated. When metabolic reactions are saturated they catalyse a constant amount of chemical per unit time irrespective of the concentration of chemical present. Graphically this may be observed as a non-linear relationship between dose and AUC, and the reaction may be described as non-linear or zero-order. Saturation of elimination may lead to prolonged retention of a foreign chemical in the body, thereby increasing its tissue concentration which could lead to increased toxicity. Saturation of a metabolic detoxication pathway may result in a shift to a non-saturated, metabolic activation pathway which could increase the formation and tissue concentration of a reactive metabolite and increase the extent of toxicity. Saturation of plasma protein binding may increase the availability of the chemical to the tissues and thus also enhance toxicity.

3. HAZARD IDENTIFICATION

3.1 Animal studies

3.1.1 Introduction

There is an increasing public demand in all societies for greater assurance regarding the safety of chemicals in the environment for which there is a potential for human exposure. Chemicals, which may occur in air, water, soil and food sources, have been the impetus for a tremendous increase in both basic research and regulatory activity in the field of toxicology. While the ultimate goal is the protection of human health, information for use in the evaluation of safety is primarily derived from a host of toxicological studies involving animal models, since direct human toxicity testing is unethical.

Toxicity studies vary widely in purpose, design and conduct ranging from relatively well-standardized and widely accepted test methods and protocols for assaying various types of toxicity to large numbers of basically research oriented investigations employing specialized study designs. Both well standardized and research oriented toxicological studies have been utilized in a broad array of risk assessment paradigms from many investigators, national and international agencies. Toxicity tests for chemicals, pesticides and drugs for which there are national and international regulatory requirements are currently performed mainly in accordance with legally prescribed guidelines. The thrust of national legislations pertaining to foods, drugs and other chemicals tends to be similar among developed countries. A major principle of regulatory toxicology requires that animal data generated for risk-assessment purposes must be biologically credible in terms of observed dose-effect relationships and should be relevant to the human setting. The European Commission (EC) and the Organisation for Economic Co-operation and Development (OECD) have international harmonization in this area; in the USA toxicity testing is generally performed according to the US Food and Drug Agency (FDA) and US Environmental Protection Agency (EPA) testing guidelines published in 1982 (updated in 1993), and in 1984, respectively, by the above two agencies. Causal relationships between exposure to an agent and various forms of toxicity can be readily established using controlled animal studies. Toxicity tests for chemicals are currently performed mainly in accordance with legally prescribed OECD and US FDA guidelines and "Good Laboratory Practice" (GLP) which covers a host of factors including housing, animal welfare and record keeping. Good Laboratory Practice for laboratory toxicity tests and Quality Control (QC) for analytical work are equally relevant to non-regulatory toxicity studies. Chemical risk assessment should utilize all relevant and available data including those from studies carried out for research or scientific investigation, e.g., academic laboratories, and the knowledge that these data are supported by GLP adds to the confidence in their use (WHO, 1992).

3.1.2 Testing strategies and methodologies

Toxicity testing is an essential part of the development of chemicals to ensure their safe use under the anticipated conditions of use and exposure. The type and extent of

testing of chemicals depends heavily on the anticipated use. A chemical used as an intermediate in synthesis may, apart from the possible contamination of the end-product, specifically have only potential occupational exposure, thus necessitating adequate testing for that specific aspect. On the other hand, when chemicals are produced to be included in food or when chemicals are expected to reach the food chain or drinking-water as a contaminant, more comprehensive testing including long-term toxicity may be necessary.

The testing of chemicals is required by regulations. Such regulations may differ between countries, and even between different government agencies in a country. However, they all require that chemical substances introduced to the human environment, directly or indirectly, must not evoke any significant hazard to humans. Nevertheless, as noted earlier in Chapter 1, only relatively few chemicals of the millions of chemicals presently known have been thoroughly tested for toxicity. For practical and economic reasons it seems to be impossible to test all chemicals in full for toxicity. Therefore, priority setting is necessary and this is usually based on two different criteria:

(1) Anticipated exposure: production volumes and levels of use, emission and waste are important aspects of the assessment of potential exposure

(2) Toxic properties: initial knowledge of certain (key) properties or structure activity relationships; properties such as acute toxicity, genotoxicity, or the potential for accumulation are important factors for setting priorities for toxicity testing

The OECD chemicals programme uses screening information data sets (SIDS) to prioritize the so-called high production volume chemicals which represent only a very small part of the millions of (man-made) chemicals presently available.

Testing strategies mainly depend on the use pattern of a chemical. Testing for genotoxicity is common for most, if not all, chemical substances. For industrial chemicals, when occasional exposure to humans is expected, the toxicity testing is focused on acute and sub-acute toxicity studies via the dermal, oral and/or inhalation routes. If human exposure is anticipated to occur more frequently, repeated dose tests of longer duration may be indicated. For pesticides, exposure may occur in the occupational setting (manufacturing and applications) or via residues in food and/or drinking-water. Thus testing for the occupational exposure necessitates acute or sub-acute testing via the dermal and/or inhalation routes, as long as incidental exposure is expected. Exposure via residues in food requires at least sub-chronic toxicity testing and, depending on duration and extent of potential exposure and the toxic nature and mechanisms of action of effects, may also warrant more elaborate testing such as chronic toxicity or tests for specific toxicity. The same approach is usually followed for chemicals intentionally or unintentionally present in food. For chemicals used in cosmetic preparations the first focus is on dermal toxicity and irritation and, when indicated, ocular or mucosal toxicity. In general, the toxicity testing of synthetic food additives and pharmaceuticals is the most extensive and includes also the need for carcinogenicity, reproductive toxicity, metabolism and kinetic data.

These above general remarks show that toxicity testing, although fitting into a general framework, should be "tailor-made" for the chemical being evaluated. A tier approach is usually advocated since it allows the producer of the chemical to withdraw it at any time in the development process, thus minimizing costs of testing if the prospect of the commercial production of a chemical becomes negative. A further advantage of such an approach is that animal experimentation is kept to a minimum.

Several decision trees for safety evaluation have been suggested by the EU (7th Amendment 1992). Recently, the US Food and Drug Administration has advocated an approach in which "concern levels" indicate the need for (further) toxicity testing. On the basis of information about molecular structure this places a chemical in three broad categories:

A for substances for which the potential for toxic effects is considered to be low,

B for those substances whose potential for toxic effects is likely to be intermediate between categories A and B.

C for substances for which the potential for toxic effects is considered to be high,

Levels of concern are identified within each category (A–C) on the basis of the anticipated exposure, and the level of concern is then used to determine the extent of toxicity testing considered necessary. For example, only short-term tests for genetic toxicity and short-term toxicity tests with rodents are advocated initially for concern level A, whereas for concern level C the full range of toxicity tests, including sub-chronic, chronic, reproduction and carcinogenicity, is advocated, as well as screens for specific toxicity such as immuno- and/or neurotoxicity.

A stepwise procedure is advised since it provides the investigator the possibility to perform specific studies at any appropriate moment, in order to identify effects at an early stage. Such effects may subsequently define and influence further testing. The ultimate goal of toxicity testing is to obtain an adequate toxicity profile of the substance in question, which may include data relating to different routes of exposure, different species, different conditions of animal health, diet, husbandry.

Studies for safety evaluation are focused on two different aims, i) the identification and definition of any possible hazard, and ii) the establishment of no-observed-adverse-effect levels to assist in risk assessment. Each aim may hinder the development of data relevant the other aim: different approaches and experimental designs are usually necessary to obtain adequate information on the nature and mechanism of action of the toxic effect compared with studies primarily designed to define the NOAEL.

Toxicity studies should be properly planned, designed, conducted, presented, and interpreted. This has been the rationale for international organizations to describe and define appropriate test methodologies. The existence of a great variety of regulations and guidelines, nationally and internationally, has been the impetus for the OECD in 1982 to develop harmonized test guidelines. Such guidelines provide the possibility to

compare studies carried out at different places and even more important, to use the same studies for evaluation in different countries, thus providing economic and, in particular, animal welfare advantages. Guidelines for testing should, however, not discourage scientific creativity.

In toxicity testing important issues include: the route of chemical exposure, selection and care of the experimental animals, environmental variables (caging, diet, temperature, humidity), careful selection of dose levels, parameters studied (end-points measured), data acquisition, presentation, and interpretation of results. The composition of the test substance should be known and should include name, composition, with quantity of components, impurities or contaminants. Additional essential information including methods of analysis, stability, some physicochemical properties, e.g., organic solvent/water partition coefficient, pK, ionization, particle size and shape, and, when applicable, density, vapour pressure and reactivity. Ideally a single lot of test substance should be used. Although it has some merit to investigate a substance in its highest purity, common practice is to investigate the technical grade product used in commerce since this is more relevant to the extrapolation to humans. The substance may be administered via the appropriate route and when administered in the diet may be given as a fraction of the total diet or at a predetermined dose level in mg/kg body weight. In this latter case the dietary concentration should be adjusted weekly or twice-weekly; twice-weekly measurements are especially important during the growth phase of the animals, when they are consuming a higher proportion of feed in relation to their body weight and there are marked temporal changes in body weight.

It should be recognized that controversy continues to be part of toxicity testing in many quarters. The economic, social and political stakes are quite high as industry spends enormous sums, estimated to be in the hundreds of billions of dollars to comply with regulations for minimizing risks to workers, consumers, and the environment, for health care for those affected by exposure to toxic materials and for litigation costs. The usefulness of animal results for predicting human risks, and whether animal tests should be employed at all are two of the contentious issues still plaguing some scientists, environmentalists, industrial representatives and government regulators.

3.1.2.1 Consideration of mixtures

Although safety evaluation of exposure to chemicals is generally based on studies of single compounds, humans are exposed to combination of chemicals. The difficulties of toxicity studies with mixtures is broadly acknowledged and a source of continued concern. The extreme complexity of mixture toxicology has suggested new research methodologies to study interactive effects (taking into account limited resources), including statistical designs and mathematical modeling of toxicokinetics and toxicodynamics. The difficulties are compounded with the inclusion of multiple end-points, including multiple organs, multiple effects, multiple mechanisms and potential interactions between such mechanisms. Combination toxicology, the toxicological investigation of exposure to multiple chemicals where the exposures can be either concurrent or temporally separated, is regarded as one of the most difficult specialties in toxicology.

Complex mixtures are more likely to produce unexpected results than are individual chemical substances. Mixtures are composed of various substances, exposure to which can be expected to be associated with different toxicities. The constituents of a mixture sometimes combine chemically to produce new compounds with different toxicities. Additionally, the presence of some mixtures might mask, dilute or increase the toxicity of other materials. Such phenomena, referred to as interactions, can amplify or reduce anticipated results. Another consideration is that different doses of separate materials might increase the bioavailability of materials that are otherwise non-toxic at the doses present in the mixture.

The potential adverse impacts of chemicals are dependent on a number of factors including; levels and duration of exposure, the mechanism of action and interactions between chemicals in a mixture. It should be noted that hazard and risk assessment of chemicals performed by regulatory agencies have primarily focused on the toxicities of individual compounds and to a remarkably diminished extent to mixtures.

3.1.2.2 Toxic equivalency factors (TEFs and TEQs)

Chemical interactions are important determinants in evaluating the potential hazards and risks of exposure to chemical mixtures. The toxic equivalency factor (TEF) approach has been extensively used for hazard assessment of different classes of toxic chemical mixtures. The overall toxicity or toxic equivalents (TEQs) of a mixture are defined by the concentration of individual compounds (C_i) in a mixture times their relative potencies of TEFs. $TEQ= \sum [C_i] \times TEFs$.

The assumptions implicit in utilization of the TEF approach include: the individual compounds all act via the same biologic or toxic pathway; the effects of individual chemicals in a mixture are essentially additive at submaximal levels of exposure; the dose-response curves for different congeners should be parallel; and the organotropic manifestations of all congeners must be identical over the relevant range of doses. The TEF values are either derived for a species-specific response or are a composite value obtained from TEFs for several responses, and individual TEFs are usually determined relative to the activity of a standard reference compound. The TEF approach has been applied to different structural classes of compounds including halogenated aromatic hydrocarbons (PCDDs and PCBs), polynuclear aromatic hydrocarbons (PAHs) and endocrine disruptors.

3.1.3 Selection of test species and general comments on test design

The selection of species and strains for toxicity studies is subject to many factors and is well documented by IARC, OECD and WHO.

Rats and mice are the most commonly used laboratory animals for toxicity testing for reasons of cost, relative ease of handling and breeding, and their full lifespan of 2 to 3 years permitting lifetime exposure to a substance. Rodents such as hamsters and guinea-pigs are much less frequently employed. Many studies are also conducted using rabbits, dogs and non-human primates such as monkeys and baboons. The choice of

animal species in many national guidelines, e.g., US EPA, and US FDA has been generally based on a number of factors including: husbandry requirements, purchase and maintenance costs, size, lifespan, characterized genetic and developmental history, and knowledge of historical records. For risk assessment purposes, the US FDA currently requires a variety of studies in rodent and non-rodent species as prerequisites for the clinical investigation and eventual marketing of new drugs, and market approval of new food additives. Short- and long-term toxicity is usually assessed in rats and dogs, reproductive and developmental toxicity in rats and rabbits, and carcinogenicity in rats and mice. However, at times another rodent species such as the hamster, or another non-rodent species such as the monkey have been used as an alternative or additional species.

Variations in biological response are found among all animal species and strains, even when the routes of exposure and frequency of the dosages employed are identical. This variability in response to chemical exposure is related to numerous qualitative and quantitative unique factors for each species. Even within species and strains, these factors can account for individual variation between members of that species. The anatomical and physiological differences between species are principally due to genetic differences manifested in biochemical variations (in digestive tract enzymes, level of circulating enzymes, liver enzymes, other degradative or detoxification processes), urine volume and pH, amount of body fat, constituents in the diet, physical activity and other stresses. The factors affecting the biological response to chemicals include: the amount, route of exposure, physical and chemical properties of the agent, absorption and excretion (distribution, metabolism, accumulation, and elimination), age, sex, health of the exposed animal species, size of the groups and individual variation. A number of metabolic differences of potential pharmacological and toxicological significance exist between rodents and humans.

Experience with, and knowledge of, the animal to be used is particularly important and ideally the animal of choice should closely resemble humans in terms of absorption, distribution, metabolic pattern, excretion and toxic action in the target tissue(s). The animals should be sufficiently sensitive, uniform in response, well characterized, available, disease free, and economically effective. In addition statistical considerations should be taken into account. Randomly bred as well as inbred strains are used, the latter being preferred in specialized toxicity tests. For logistic and economical reasons usually small laboratory rodents (mostly rats and mice) are used, but for specialized toxicity testing many other species may be used. Retrospective analysis of toxicity tests has indicated that sex-related differences occur in half of the tests, and these are decisive in the establishment of a no-effect level, clearly indicating the need to use both sexes in toxicity tests. After selection of the animals, transportation, logistics, quarantine, disease surveillance and random allocation to the experimental groups need close attention and adequate documentation.

The diet may have a considerable impact on the toxicity test results. Acute toxicity in fasted animals, for example, is generally more severe as compared to non-fasted animals, and may differ 2–3 fold by oral exposure and up to 10 to 20 fold by inhalation. Diet composition influences physiology and different levels of macro- and

micronutrients influence biotransformation and enzyme activities. Over-nutrition (easily attained by the present day practice to feed animals *ad libitum*) may cause diseases such as progressive nephropathy, cortico-medullary nephrocalcinosis, or endocrine disturbances. Food restriction reduces these confounding effects and reduces at the same time spontaneous and induced tumour incidence in long-term experiments (Roe, 1988; Lok et al., 1990). High dietary concentrations may influence palatability of the feed, and/or may alter the feed composition. Diet and drinking-water should always be controlled for the presence of (naturally occurring) toxins and contaminants. Both diets made from natural ingredients and purified diets (the latter usually contains refined proteins, carbohydrates and a vitamin and mineral mixture of high purity) are used and both have their advantages and disadvantages.

Cage grouping, bedding, temperature, humidity, lighting and noise may all influence experimental animal response. Specifications for housing and care are included in test guidelines of the OECD, IARC and WHO. In recent decades the number of parameters to be studied has increased considerably, due to a better understanding of physiology, biochemistry, and pathology. Clinical chemical and histopathological measurements have increased considerably, although this increased number of parameters does not necessarily assure more relevant information. It may even give the toxicologist the false sense of confidence and there are good reasons to re-evaluate their relevance. Endocrine and immunological parameters are also increasingly utilized. However, for example, atherogenic indicators, which would be particularly relevant to human health risks are, in the main, absent in assessment schemes.

Information and automation technology permit the use of integrated computerized data storage and retrieval systems. For current standard toxicity testing they are almost a necessity, especially since they provide for the optimal assurance required by internationally accepted Good Laboratory Practice (GLP) regulations. However, automatic data processing should be validated and quality controlled. The results of toxicity tests should be reported in detail, including the presentation of individual data (OECD test guidelines). According to the principles of GLP, changes in design, experimentation, environmental variables, or clinical signs should be recorded adequately. Histopathological reporting, including the number of animals and organs examined should be detailed and tabulated per group per sex per organ.

Many aspects are involved in the process of interpretation and evaluation of results and only some general remarks follow. Clear and objective interpretation of results is related to as precise a definition as possible of the experimental objectives, the design, and conduct of the study. Depending on the studies performed, information on the no-observed-adverse-effect level (NOAEL), as well as information concerning the nature of the toxic effect, the dose-effect and dose-response relationships, may be obtained. All the data on measured parameters (weight, weight gain, body weight plotted against time, absolute and relative organ weights, food and water intake, biochemical and haematological effects, clinical signs and histopathological changes) are relevant for the evaluation. Investigators should be aware of confounding factors, and statistical significances which may or may not be relevant (at $P < 0.05$ one out of 20 tests of parameters may be expected to be "significant"). Although investigators should use

common sense, and a critical mind it is possible that considerable differences in interpretation occur. Independent peer review of results is a well accepted procedure nowadays. As long as interpretations are well described and reasoned such differences are acceptable and will not discredit toxicological science.

Non-clinical toxicity studies that are part on a safety submission for marketing or regulated products are required to be carried out according to the principles of Good Laboratory Practice (GLP). Such GLP regulations became necessary because there were cases of malpractice in producing toxicity data. Quality measures encompass two elements, quality control and quality assurance. Quality control maximizes the accuracy and validity of data. Quality assurance is the technique followed (i.e., inspections and audits) to verify that design, planning, procedures and quality control are adequately carried out. GLP regulations also require the use of qualified personnel. On the job training in order to obtain practical experience, in addition to the education process, is a necessity. In most industrialized countries, accreditation procedures have been implemented. Compliance with GLP facilitates international acceptance of studies and prevents duplication. However, GLP regulations are not in themselves proof of the best possible experimentation.

The use of animals for experiments has been under increasing scrutiny for many years. Increasing use of vast numbers of animals and the levels of discomfort for experimental animals prompted animal welfare organizations to criticize severely toxicity testing procedures, in particular and with some justification, tests such as the LD_{50}, and LC_{50}, eye irritation, and skin irritation. It is the responsibility of every toxicologist to reduce as far as possible the number of animals to be used, to minimize stress, pain and discomfort and to search for adequate alternatives such as those directed to the use of *in vitro* techniques.

3.1.4 Single dose acute toxicity

Acute toxicity is defined as the adverse effect(s) occurring within a short time (usually within 14 days) after administration of a single dose (or exposure to a given concentration) of a test substance, or to a divided dose given within 24 hours. (Some laboratories include treatment periods of 14–28 days under the description of acute toxicity, but others differentiate such a test as short-term testing). Acute toxicity provides preliminary information on the toxic nature of a substance for which no other toxicology information is available. Before testing a substance, its chemical and physical characteristics including molecular weight, partition co-efficient, and the toxicity of related compounds should be considered carefully before determining the dose, otherwise oral toxicity, including lethality caused by inappropriately large doses of a chemical may have no biological relevance. The classical acute toxicity test involves the determination of a median lethal dose via the route of administration relevant to human exposure. Descriptions of the LD_{50} (oral or dermal) or LC_{50} (inhalation route) are available in the OECD Test Guidelines No. 401 (oral), No. 402 (dermal), and No. 403 (inhalation). The LD_{50} is the statistically derived expression of a single dose that can be expected to be lethal to 50% of the animals. The principle of an LD_{50} test is that groups of experimental animals are administered graduated doses via the oral (via gastric

intubation) or dermal route, and examined for signs of toxicity and/or death. Animals dying during the test and surviving animals are autopsied for gross examination. Histopathological examination is only performed when indicated. LC_{50} is the concentration of a substance in air that causes death following a certain period of exposure. In this type of study the exposure is defined in terms of concentration (c) and the time (t) and is sometimes expressed as the product c.t. In routine practice toxicologists usually keep the time constant (i.e., 1 or 4 hours) and vary the concentration of the test substance. The observation period for acute toxicity tests is 14 days.

The use of LD_{50} or LC_{50} tests has been increasingly seriously questioned, especially the need for statistical precision of the lethal dose, necessitating the use of large numbers of experimental animals, and in some countries has been abandoned for regulatory purposes. From the point of view of the toxicologist, the precision of the LD_{50} is irrelevant and superfluous. Efforts to develop better, more sensible, tests for acute toxicity have led to the acceptance of the "fixed dose" method (as described by Van den Heuvel et al., 1990). OECD Test Guideline No. 420, which is a very suitable alternative in which acute toxicity is tested in a stepwise fashion, using a considerable smaller number of animals, and limiting the inconvenience to animals because toxicity rather than lethality is the principal end-point. This study is also designed to provide sufficient information to classify substances according to their toxicity. In a preliminary study, various doses are administered to single animals of only one sex in a sequential manner. This "sighting study" provides information on the dose-toxicity relationship and the estimated lethal dose and usually does not require more than 5 animals. The main study is then performed with groups of 5 animals of each sex at preset dose levels (e.g., 5, 50, 200 or 2000 mg/kg body weight) with the higher doses derived from the sighting study as doses likely to produce evident toxicity but not death. When in the main study, evident toxicity but no mortality is observed, no further study is needed. When initial doses chosen do not produce evident toxicity, the next higher preset levels should be used, in contrast to the situation that occurs when at the initial doses animals die or have to be sacrificed due to severe toxicity, and the next lower preset dose levels are chosen for study. Evaluation, interpretation, and classification according to the European Union scheme are shown in Table 7.

Another alternative to the LD_{50} test is the acute toxic-class-method proposed by Schlede et al. (1992) and Diener et al. (1994), which is still based on mortality as an end-point, but uses substantially fewer animals, and acute toxic phenomena other than death can be included. An OECD guideline for this test is in preparation. A new design for acute inhalation toxicity testing was suggested by Zwart et al. (1990, 1992). By exposing pairs of rats for different periods of time to about four different test concentrations in a nose-only exposure unit, LT_{50} values (50% mortality exposure time) can be obtained for five pairs per concentration. The mortality data of the approximate 20 time-concentration combinations are then used to calculate the probit relationship. With this procedure, LC_{50} values are obtained over a 10–50 fold range in time. Moreover, the obtained relationship contains considerably more valuable information for risk assessment than a single LC_{50} value obtained by the normal procedure.

Table 7. Evaluation and interpretation of results of acute toxicity test - fixed dose procedure

Dose	Results	Interpretation
5 mg/kg body weight	Less than 100% survival	Compounds which are VERY TOXIC
	100% survival; but evident toxicity	Compounds which are TOXIC
	100% survival; no evident toxicity	See results at 50 mg/kg
50 mg/kg body weight	Less than 100% survival	Compounds which may be TOXIC or VERY TOXIC. See results at 5 mg/kg
	100% survival; but evident toxicity	Compounds which are HARMFUL
	100% survival; no evident toxicity	See results at 500 mg/kg
500 mg/kg body weight	Less than 100% survival	Compounds which may be TOXIC or HARMFUL. See results at 50 mg/kg
	100% survival; but evident toxicity	Compounds considered as having no significant acute toxicity
	100% survival; no evident toxicity	See results at 2000 mg/kg
2000 mg/kg body weight	Less than 100% survival	See results at 500 mg/kg
	100% survival; with or without evident toxicity	Compounds which do not have significant acute toxicity

OECD Test Guideline no. 420

3.1.5 Sub-chronic toxicity testing (repeated dose)

Testing of chemicals in repeated dose studies is an effective way to identify their toxic properties. To perform such studies with an appropriate dose range more information may be needed than is available from acute toxicity data. Where indicated, "dose-ranging" studies are preliminarily performed in order to define more precisely the doses to be used in a sub-chronic test. Short-term repeated dose studies, lasting 14–28 days (often referred to as sub-acute tests), using young adult animals have acquired increased utility as a bioassay with extended clinical and pathological investigations (OECD GL No. 407). When such a study is properly planned and designed, and critical, relevant, parameters are studied, the results provide a fair basis for a first toxicological evaluation in a limited time framework and at relatively lower cost.

The design of repeated dose studies may vary, but usually consists of the repeated administration of a series of 3 to 4 doses, with an increment of 2–10 between doses, to groups of 5–10 animals per sex for 14 to 28 days. The use of increments of 10 can, however, lead to quite imprecise estimation of effect levels. A non-dosed control group should also be included and treated in an analogous manner to the test group. For dosing in the diet and dermal dosing, the administration is daily, whereas inhalation exposure is usually limited to five times a week. In gavage studies, as well as in certain inhalation

studies (where exposure is limited for logistic reasons to 5 times a week), it is necessary to use an additional control group receiving only the vehicle in which a substance is administered. From the dose range chosen, the highest dose should be a clear adverse effect level with, by preference, either no or limited, mortality. The lowest dose level should not produce any evidence of toxicity whereas ideally the medium dose(s) should produce minimal (and intermediate) observable adverse effects.

The animals in a test are inspected daily for clinical signs and body weight and food consumption are monitored (usually weekly). Clinical haematological and a broad range of standard biochemical parameters are determined at the end of the test period. Where indicated other parameters such as lipids, hormones, methaemoglobin, and cholinesterase activity are measured. Urine analysis is usually included as well.

At termination, extensive gross necropsy should be performed, weights of the major organs are determined, and organ and tissues preserved for histopathological examination. Histological examination is usually performed on all preserved organs and tissues of highest dose animals and controls. Where indicated, organs and tissues of intermediate dose and low dose animals also need to be studied.

Sometimes satellite groups are included for special studies (reversibility, determination of concentration of the chemical or its metabolites in tissues or organs and the like). As indicated earlier, proper reporting and evaluation of the results are of decisive importance since they should provide regulators a clear insight into the conduct and results of the experiment. Extrapolation from the results of repeated dose studies to humans is valid to a limited degree and results may provide useful information concerning target organ(s) and mode of action of a substance.

A sub-chronic study where exposure is lasting 90 days (OECD GL No. 408) is usually designed to be sufficient in itself for a toxicological evaluation, as well as providing sufficient information for the design of chronic studies. A sub-chronic study should provide information on the major toxic effect of the test substance and indicate the target organ(s) affected. Additionally, investigation of the latent period of the development of an effect related to dose, the reversibility of an effect upon cessation of administration (usually studied in a satellite group), or tissue and blood levels of the substance or its major metabolite(s), may be performed.

Finally a 90-day sub-chronic test should provide an estimate of a no-adverse-effect level of exposure. The 90-day test is carried out with weanlings, thus including the growth phase to maturity. This period of life is considered to be particularly sensitive to exogenous agents. Sub-chronic 90-day tests possess certain limitations for human hazard assessment. Since the exposure takes place only for a certain period (approximately 10%) of the lifetime, although this is a very vulnerable period, this type of test may not adequately predict chronic effects since the substance may produce different toxic responses when repeatedly administered over a longer time. Additionally, the ageing process may alter tissue sensitivity, metabolism and/or physiological capability. Spontaneously occurring diseases may also influence the degree and nature of the effect. (Additionally, it should be noted that satellite groups in 90-day studies are

sometimes used to investigate toxicokinetics and metabolism and the reversibility of any effects).

The sub-chronic toxicity test employing 10 animals of each sex should consist of 1 control and 3 dose groups. A top dose should be chosen to indicate clear toxicity, and the lowest dose should ideally show no adverse effects. For substances of low toxicity it is important to ensure that when administered in the diet the quantities of the substance do not interfere with normal nutritional needs. When the substance is given orally it can be administered in the diet or drinking-water, or by gavage. In the latter case it is important that the administration by gavage takes place at similar times each day and that the quantity is adjusted regularly (weekly or bi-weekly) so as to maintain a constant dose level in terms of changing body weight.

Clinical observation should be carried out daily and include cageside observations such as changes in skin and fur, eyes and mucous membranes, clinical sign of respiratory, circulatory, central nervous system or behavioral changes.

At the end of the test period, the full range of haematological parameters are determined as well as clinical biochemical parameters; electrolyte balance, carbohydrate metabolism, organ function parameters, serum enzymes, albumen, protein, urea nitrogen, creatinine, and other determinations considered to be relevant for toxicological evaluation.

All animals (including those dying intercurrently) should be subjected to full gross examination including the examination of all orifices, the external surface of the body, and the cranial, thoracic and abdominal cavities and their contents. The weights of organs are recorded and all organs and relevant tissues are preserved for possible future histopathological examination. Extensive histopathological examination should be performed on all preserved tissues of the highest dose level and control groups. The organs in which lesions different from controls are found at the highest dose should also be examined in the intermediate dose(s) and lowest dose groups. Tissues or organs showing gross or clinical abnormalities should also be examined histopathologically. Sub-chronic toxicity tests usually cannot determine the carcinogenic potential of a test substance, neither will they all be able to reveal non cancer effects that require long term exposure (i.e. cataract).

3.1.6 Chronic toxicity testing

Substances which are expected to give rise to continuous human exposure or exposures for relative long periods of life are also tested in chronic toxicity tests (OECD GL No. 452). A chronic toxicity test separate from a carcinogen bioassay will rarely be carried out but for purposes of completeness this type of test is described below.

Chronic studies are usually the basis for risk assessment and establishment of "safe" human exposures such as the acceptable daily intake (ADI), hence it is usual to select doses that span the range from no-effect to a clear effect. Chronic studies may provide important clues to potential modes of action and the relevance of animal tumour

findings to humans. Hence important factors to consider include tumour types, e.g., those responsive to endocrine influence or those produced by reactive carcinogens: number of tumour sites, sexes, studies and species showing effects or no effects, influence on route of exposure; the spectrum of tumours (local or systemic sites) and target organ or system toxicity.

The duration of a separate chronic toxicity test has been under debate for many years and different requirements (from 6–24 months) exist in different countries. The duration of chronic toxicity studies is usually 12 months or more in dogs and typically 2 years (104 weeks) in rodents. The latter are often referred to and thought of as "lifetime studies" and serve this function since the duration is sufficient to allow the generation of lesions found in geriatric animals such as cancer. Two year studies avoid the excessive mortality and degenerative changes that would occur in a true "lifetime study" and would reduce the sensitivity of the test for end-points other than cancer.

In specific cases, for example, when toxicokinetic studies indicate that a steady state will not have developed a reasonable period before the proposed end of the study or when proliferative changes are found, a longer test duration should be considered. In a chronic toxicity test, the toxicity profile of a substance can be characterized, the major chronic effects noted, and dose-response relationships can be determined. General toxicity parameters, including neurological, physiological, biochemical, haematological and exposure related morphological effects can be detected. It is advisable to obtain chronic effect data in two species, preferably a rodent and a non-rodent, in order to gain insight in species variation. Based on previously conducted studies (i.e., sub-chronic data in another species), testing with one single species may sometimes be sufficient and provide enough data to assess the hazard of a substance in humans. In chronic toxicity testing using rodents each group should consist of at least 20 animals per sex (for non-rodents a minimum of 4 animals per sex per group is recommended). The addition of satellite groups may be considered. Test design is most extensive in chronic toxicity testing. Housing conditions and diet should be such that (infectious) diseases are not likely to occur, which is facilitated by the use of specific pathogen free (SPF) animals. In these types of tests individual housing is preferred and environmental exposure and contamination to the substance should be avoided. While individual housing prevents cannibalism, provides the possibility of adequate measurement of food and water intake and allows the animals to have its exercise, group caging has been claimed to reflect better the social circumstances of the animals. When the test substance is of low toxicity, or is itself a nutrient, attention should be paid to the dietary composition, since such a substance may be included in the diet at extreme high dietary levels. Except for specific reasons, such as studies with nutrients, the highest dose level should never exceed 5% of the diet, since higher levels may affect the animals nutritional requirements. Oral dosing in the diet is always on a 7-days per week basis, whereas in gavage studies and inhalation studies, dosing/exposure is usually 5 times a week; for inhalation studies, exposure is usually for a period not exceeding 6 hours a day. In gavage studies, as well as in certain inhalation studies, it is necessary to use an additional control group receiving only the vehicle in which a substance is administered. At least 1 control and 3 dose levels should be used, in which the highest dose should elicit some signs of toxicity, and the lowest dose should ideally be a no-effect-level.

During a test, careful clinical examination should be made daily, and animals inspected at least two times a day to minimize the loss and autolysis of animals which have died. Clinical signs should be recorded carefully in order to obtain data concerning the onset and progression of the toxic effects. Body weight should be determined at relevant intervals (weekly during the first 13 weeks, and monthly thereafter). Food intake should be recorded weekly during the first 13 weeks, and then at least once every 3 months.

Haematological parameters (haemotocrit, haemoglobin concentration, erythrocyte count, total and differential leukocyte count, clotting potential) should be measured before the onset of the study, at six months, and then at 6 month intervals. At the end of the test the same parameters should be measured in at least 10 animals per sex per group and, in addition, a differential blood count should be performed in the highest dose and control group, and, where indicated, at other dose levels. Urine analysis (appearance, volume, density, pH, protein, glucose, ketones, occult blood and sediment in semi-quantitative terms) and extensive clinical chemistry should be performed at the same intervals noted for haematological examination. Gross and histopathological examination should be performed as described under sub-chronic toxicity testing. It is not possible to know unambiguously or *a priori*, whether all the above parameters would be particularly informative, at the expense of less frequently studied parameters; for example, the lack of any parameter giving information on the effect of the test substance on the cardiovascular system, and the rarely studied endocrine and immune parameters.

3.1.7 Toxicokinetic studies (see also Section 2.2)

These studies involve single or repeated exposure and are used to elicit how and at what rates a chemical enters the body, is distributed throughout the body, what metabolic changes the agent undergoes within the body and how it is excreted. Toxicokinetic data can be used to predict plasma concentrations, target tissue doses, and the fate of the administered dose, which should assist in deciding which toxicity studies should be conducted, doses for chronic toxicity and carcinogenicity studies, elucidation of the mechanism of toxicity, and assist in the interpretation of toxicity studies. Toxicokinetic studies have greater relevance when conducted in both sexes of young adult animals of the same species and strain used for other toxicity tests with a test substance. The number of animals used in these studies should be sufficient to estimate reliably population variability. Metabolic and toxicokinetic parameters are usually determined following a single administration of a test substance, and multiple dosing studies can be employed to determine the potential of a compound to alter its absorption, distribution, metabolism, or excretion. *In vitro* studies can also be useful since they can usually indicate identical metabolic pathways and comparable metabolism rates to those obtained from whole animal studies.

3.1.8 Skin and eye irritation and sensitization

Evaluation of materials for their potential to cause dermal irritation and corrosion has been common since the 1930s for industrial and agricultural chemicals, cosmetics and consumer products. Currently all the established test methods, which are basically

those proposed by Draize et al. (1944), use the same animal model, the rabbit (almost exclusively New Zealand albino), though other animals have been proposed by Gad & Chengelis (1988). Testing is performed to evaluate the potential to cause skin irritation, characterized by erythema and oedema. The severity of irritation is measured in terms of these parameters and their persistence.

There are 3 types of irritation tests:

a) primary (or acute) irritation (localized reversible dermal) resulting from a response to a single application of, or exposure to a chemical, is the most commonly used study;

b) in special cases, cumulative irritation (reversible dermal response from repeated exposure to a substance);

c) photochemically induced irritation (a primary irritation resulting from light induced molecular changes in the chemical to which the skin has been exposed).

Most regulations and common practice characterize as not reversible an irritation that persists 14 days past the end of exposure. The primary eye irritation test is intended to predict the potential for a single splash of a chemical in the eye of a human to cause reversible or permanent damage. There are many regulatory guidelines for ocular irritation test methods all employing the New Zealand albino rabbit. There have been a number of modifications, or tiered designs, proposed since the introduction of the Draize test 50 years ago. The OECD methods for skin and eye irritation involve a hierarchical screening process to obviate the need for testing severe irritants. In order to avoid testing severe irritants or corrosives in animals a test substance will not be studied for eye irritation if the substance is a strong acid (pH 2 or less), or strong alkali (pH 11 or greater) and/or if the test substance is a severe dermal irritant (with a Primary Dermal Irritation Index of 5–8) or causes corrosion of the skin.

The determination of a substance's skin sensitization potential (allergic contact dermatitis) provides information on a potential human hazard likely to arise from repeated dermal exposure. Once an individual has been sensitized, a symptom may follow any subsequent skin exposure to the allergen, and, at times may even following ingestion or inhalation exposure. Allergy or hypersensitivity is determined by a topical application or intradermal challenge injection of the chemical to young albino guinea-pigs, given after a sensitizing treatment(s) which produces a local response greatly in excess of that produced in non-sensitized animals. Guinea-pigs are selected for skin sensitization studies, since they are known to elicit reactions similar to those that occur in humans and additionally a substantial amount of background laboratory data has been accumulated for this species.

Skin sensitization tests can be classified systematically into two types:

1. Involving the concomitant use of Freund's Complete Adjuvant (FCA), an immunopotentiator, to facilitate induction of sensitization. Among the test methods involving the use of an adjuvant the most prominent are:

i) the Magnusson and Kligmann maximization test (Magnusson & Kligmann, 1969);
ii) the adjuvant and patch test.

The Magnusson and Kligmann maximization test combines FCA, sodium lauryl sulfate, intradermal injection and occlusive topical applications of the test material during the sensitization period.

2. Test methods not incorporating the use of an adjuvant (Draize test, Buehler test, and the open epicutaneous test are also employed for evaluation of the intensity of positive responses that have been noted in a test with the concomitant use of adjuvant.

Substantial interpretative knowledge is required to minimize the margin of error in extrapolating animal allergic contact dermatitis to probable human experience. For example, a positive result may be due to the presence of a trace impurity in an amount which can vary from batch to batch and which is a potent sensitizer.

3.1.9 Carcinogenicity testing

It is now generally accepted that the induction of cancer in animals and man involves several consecutive independent events. The process of carcinogenesis is described in 2.1.3.3.

In the cancer bioassay both genotoxic as well as non-genotoxic carcinogens can be detected since the end-point in this assay is the development of cancer. Since according to some authorities it is assumed that non-genotoxic carcinogens operate through mechanisms which have biological threshold, which is a rationale for regulatory decision making to adopt a NOAEL uncertainty factor approach, it is important to understand a substance's genotoxic or non-genotoxic potential (see 2.1.3.3). Genotoxicity testing in *in vitro* assays are described in the Section 3.2.

Carcinogenicity bioassays are performed when prolonged or continuous exposure to humans is likely. These are usually carried out after a chemical has been tested in a battery of short-term genotoxicity tests (see 3.1.1 1). For new chemicals, depending on the results of a battery of short-term tests and on anticipated use, further testing may not be necessary.

A new chemical which in a short-term battery with various end-points is found to be genotoxic will usually not be accepted for human exposure, thus making further testing usually unnecessary, unless either the use or exposure are absolutely essential or unavoidable. In these cases a carcinogen bioassay is warranted to obtain information on carcinogenic potential and dose-response relationship in order to carry out a quantitative risk assessment. For non-genotoxic substances carcinogenicity testing is usually required although the risk would be expected to be minimal when exposure to humans to such substances is far below (i.e. x 1000 or less) a no-observed-adverse-effect level (NOAEL).

When non-genotoxic substances are investigated it is also relevant to study the tumour enhancing (promoting) properties. Promotion is an organ-specific phenomenon, and thus such a study should be focused on lesions found in toxicity studies in target organs (e.g., hyperplasia or increased cell turnover). "Limited" *in vivo* assays are extremely useful as indicators of possible tumour enhancing properties and available systems include skin, lung, breast, liver, forestomach, colon, and bladder.

3.1.9.1 Carcinogen bioassay

Carcinogenicity bioassays are generally conducted for 3 basic reasons: 1) the experiment may be part of a screening program for the detection of potential carcinogens, 2) the experiment may enable estimation of risks at low levels of exposure and 3) the experiment may allow verification of scientific hypotheses about the mechanisms of carcinogenesis.

Although a carcinogenicity test specifically designed to detect carcinogenicity can be performed, a combined chronic toxicity/carcinogenicity bioassay, in which both neoplastic and/or non-neoplastic effects can be determined is more commonly employed.

For the past 4 decades, assessment of the carcinogenic potential of chemicals has largely relied upon the interpretation of chronic bioassays conducted in rodents. Despite steady improvements and standardization of study design and conduct, interpretation and extrapolation of these experimental results to human carcinogenic risk is often difficult and contentious.

IARC and the National Toxicology Program recommend rats and mice of both sexes should be used. The bioassay is commenced with weanling or post-weanling animals and covers the animals' lifespan of at least two years (rats) or 18 months (mice). Since information concerning dose-response is crucial, sufficient dose groups should be used. At least three dose levels and a control group, and at least 50 animals per sex per group, should be used. The lowest dose should not interfere with growth and development and must not cause effects whereas the highest dose should produce signs of toxicity. The highest dose should not exceed a concentration of 5% unless macro-nutrients are being examined. The intermediate dose should be in a mid-range between the high and low doses. Occasionally a satellite high dose group (20 animals per sex) to induce frank toxicity, and a satellite control group (10 animals per sex) for evaluation of the pathology of other effects than neoplasia (usually after 12 months experimentation) may be added. As described previously under chronic toxicity testing, caging, care, diet and water supply are to be optimal and well controlled. It is important to note the issue of animal survival. A tumour incidence for a specific tissue site may be lowered at high doses, because the animal does not live long enough (due to other tumours or other pathologies) to develop tumours occurring late in life.

The carcinogenesis bioassay employed by the National Toxicology Program (NTP) in the USA employs a maximum tolerated dose (MTD), another dose equal to one-half or one-fourth of the MTD, and an untreated or vehicle control.

The maximum tolerated dose has been traditionally defined as the maximum dose administered for a major portion of a laboratory animals' lifetime that can be tolerated without resulting in significant impairment of growth (e.g., no more than 10% impairment or reduction) or without producing observable toxic effects (other than carcinogenicity) that would significantly shorten the animals' lifespan. The MTD should not cause morphological evidence of toxicity of a severity that would interfere with the interpretation of bioassay. In general, the low dose selected for carcinogenicity tests should not be less than 10% of the high dose.

The MTD is used to give the greatest chance of observing an effect of the chemical in animals. (The combined results of over 400 bioassays conducted by the NTP show that exposure to a MTD of the chemical for over two-thirds of the animal's life span is needed for even DNA-reactive agents). However, many have questioned its use since high dosing occurring at the MTD may increase the number of tumours. Gold in 1998 noted that "near-toxic" doses can frequently cause cell death due to cell killing and consequent cell replacement, and these effects can be unique to high doses. "Cell division itself can increase the chance of mutations and tumours and then the effects at low doses are likely to be much less than a linear model would predict and may often be zero. Testing for carcinogenicity in animals at near toxic doses does not predict the excess number of cancers from low doses typically experienced by humans".

Exposure to the substance should optimally be as comparable to the anticipated exposure of humans and the frequency of exposure is usually dependent on the route of exposure. In oral studies the substance is given daily, unless the substance is given by gavage, in which case exposure is usually restricted to 5 times weekly. In inhalation studies, exposure will usually be limited to 6 hours per day 5 times a week. Careful daily clinical examination is required and appropriate actions should be taken to minimize loss of animals during the study due to autolysis and/or cannibalism. Body weights are measured weekly during the first 13 weeks and thereafter once every 4 weeks. Food and drinking-water intake are determined during the first 13 weeks and thereafter at 3 monthly intervals. Haematological examinations are performed after 3, 6, 18 and 24 months on 20 animals per sex per group and a differential blood count is performed on samples of animals of the highest dose group and controls and in lower dose levels when indicated. Urine analysis in 10 animals per sex per group should be performed at the same time interval. At 6-month intervals clinical chemistry to the same extent described for chronic toxicity testing should be performed. At the end of the bioassay 50% survival for mice at 18 months and rats at 24 months is expected. Complete gross examination is performed and histopathological examination is carried out on all tissues and organs from the highest dose and control group. Where indicated, tissues and organs of lower dose groups should be examined and all tumours or lesions suspected of being tumours should be examined histopathologically.

It should be stressed that the rodent species and strains selected for carcinogenicity studies should preferably possess low incidences of spontaneous tumours. Additionally it is important to consider the test animals' general sensitivity to chemical carcinogens and the responsiveness of particular organs and tissues of test animals to carcinogens. Although, currently, there is no scientific basis for selecting among inbred, out-bred or

hybrid rodent strains for carcinogenicity studies, a major consideration is that the selected test animals are derived from well-characterized and healthy animals. It should be noted that the use of highly specific inbred strains for the 2-year rodent bioassay can give rise to high strain-specific background tumour incidences, and chemical-specific tumour responses (in single species and/or a single tissue) that might be irrelevant to humans. Proposals have also been made to perform the carcinogen bioassay only in male rats and female mice. In the past, many toxicological guidelines (e.g., US FDA, 1982) suggested standards for valid negative carcinogenic bioassays that required 50% survival of rats until 24 months of age. However, studies from the National Toxicology Program and industrial laboratories suggested difficulty in reliably achieving 50% survival at 24 months. The important issue of the connection between tumour incidence at some tissue sites and body weight should be noted. An association of leukaemia, pituitary, and mammary tumours with body weight in rats and between body weight and liver and lung tumours in mice was demonstrated by Hasemann & Rao (1992) and Hart & Turturro (1994) observed reduced tumour incidence at these sites with calorically restricted animals. Different body weight in different dose groups can affect comparisons of tumour incidence, indicating a need for diet control in chronic bioassays or adjustments for the confounding effect of body weight differences.

The US National Toxicology Program has directly or indirectly provided a large component of the basic scientific data including the 2-year studies that other federal and state scientific and regulatory agencies use to protect human health. Although broadly considered the best test model to date for obtaining carcinogenicity data, the 2-year rodent bioassay is limited because it uses large number of animals, is expensive to operate and normally takes 5 to 8 years to complete and evaluate before results are reported, after which results must be extrapolated to humans.

Ashby in 1996 noted that the US NTP had evaluated the carcinogenicity of about 400 chemicals over the past 20 years at a cost of hundreds of millions of US dollars and suggested alternatives to the 2-species bioassay for the identification of potential human carcinogens. In the large majority of cases a practical estimation of the carcinogenic potential can be formed in the absence of lifetime carcinogenicity bioassay data. This can be achieved by a sequential study starting with an appreciation of its chemical structure and anticipated reactivity and mammalian metabolism. After the short-term evaluation of a range of additional properties of the agent including its genetic toxicity, confident predictions of the genotoxic and/or non-genotoxic potential of the agent can be thus made.

A relational retrieval data base was developed by Calabrese & Blain (1999) compiling toxicological studies that assess whether a single dose of a chemical or physical agent, without exogenous promotional stimuli, could cause tumour development in animal models. This data base allows for an evaluation of these studies over numerous parameters important to tumour outcome which includes type and quality of the studies as well as physical/chemical properties of the agents. An assessment of the data base, which currently contains approximately 5500 studies involving about 800 chemicals from 2000 articles, reveals that a single dose of an agent can cause tumours to develop in males and females of numerous animal models in all

principal age groups. Additionally, the range of the 426 agents causing a positive response is chemically diverse, with representatives from over several dozen chemical classes. The findings indicate that the phenomena of single-exposure carcinogenesis is widespread and highly generalizable across chemical class, route, dose range, species, age and gender. Single-exposure carcinogenesis requires a careful and formal consideration especially as it may pertain to accidental spills, leaks, fires, explosions and exposure excursions. How this information may be utilized in the process of risk assessment is yet to be determined unambiguously.

3.1.9.2 Transgenic mouse models

Transgenic rodent models have recently emerged as potentially useful tools for facilitating the more rapid detection of genotoxic carcinogenic chemicals and strengthening the interpretation and regulatory utility of chronic rodent toxicity and carcinogenesis studies. Transgenic animals possess genetic alterations such as the presence of specific oncogenes or absence of tumour suppressor genes that are critical to the multistage process of tumorigenesis, but by themselves are insufficient to induce cancer. Two tumorigenic lines, Tg.AC and heterozygous $p53^{def}$ deficient mice have been found by the National Institute of Environmental Health Sciences (NIEHS) in recent studies in the USA to offer a battery of experimental methods that could be routinely employed for the assessment of carcinogenic potential. Additionally, if within the short time period of the assay (about 6 months), the presence of the transgene itself does not result in spontaneous tumour induction. This will avoid one of the major confounding factors of the conventional bioassay. Hence a short-term carcinogenesis bioassay can permit the more facile differentiation between age-related carcinogenesis and chemical carcinogenesis.

3.1.9.3 Limited in vivo *bioassay*

Numerous protocols have been devised to demonstrate that specific agents are carcinogenic without the expense and the time required for the full carcinogenesis bioassay. Most of these tests involve determining tumour information at one site only and therefore cannot fully reflect the carcinogenicity of an agent. The earliest examples in this area were the Strain A mouse lung tumour test of Shimkin & Stoner (1975), and the bladder implantation technique of DeSesso et al. (1987). Tests such as these are further limited in their usefulness by the fact that animals not given a test chemical develop high yields of tumours. This makes an appreciation of the significance to human health of induced or accelerated yields of tumours, such as those induced by these techniques, exceptionally difficult.

The second approach has been to adopt the initiation-promotion type protocol in which a time-limited dose of a potent carcinogen is followed by the administration of the test agent. The initiating carcinogen as noted by Ito et al. (1990, 1992) may be either tissue-specific or more widespread in its range of action. This process measures only the tumour promoting effect of the test agent and therefore, while of considerable academic interest in determining the mode of action of a carcinogen, is of little value as a primary carcinogenicity screen.

A more promising recent approach is the study of preneoplastic lesions or foci in tissues such as the rodent liver. Presently, this suffers from the disadvantage that, in many tissues, identified possible preneoplastic lesions lack the certainty that they will progress to frank malignancy. This has demonstrated with some degree of certainty only in a limited number of tissues. Again this indicates the dangers in using such an approach in place of, or as a prelude to, a full carcinogenesis bioassay.

3.1.10 Genotoxicity testing

As discussed under 2.1.3.3 and this section genotoxicity testing is a widely recognized as a very relevant prescreen for potential carcinogenicity as well as teratogenic potential. In addition, these tests serve the purpose of establishing whether chemicals have the potential or not to induce cell mutations at the gene and/or chromosome level. A number of such tests have been described, e.g., OECD, Weisburger & Williams (1991) and by the UK Department of Health Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (UK COM) in 1991. More than 70 different methods have been reported with a number of different genetic end-points. The tests are generally subdivided on the basis of the end-point measured and the phylogenetic levels represented.

The strengths and weaknesses of short-term genotoxicity tests have been discussed by many authors and have been well described by Ashby et al. (1990). As indicated earlier, genotoxicity tests do not detect all carcinogens since (human and animal) carcinogens as such can be divided into those which interact with DNA and those where the carcinogenic action is caused by a different mechanism of action not involving an interaction with DNA (see also 3.1.9). Thus genotoxicity testing provides only information concerning possible genotoxic potential, and while chemicals with this potential are suspected of being carcinogenic, the final proof can only be derived from animal experimentation.

A correlation between animal carcinogenicity and genotoxicity can only be present for those animal carcinogens which act via an interaction with DNA. Therefore correlation studies involving all animal carcinogens and *in vitro* genotoxicity tests are, by definition, false. For this reason "detection" rates for carcinogens in *in vitro* systems vary from 75 to 45%, depending on the number of non-genotoxic carcinogens included in the study. Nevertheless genotoxicity tests as a prescreen are generally acknowledged to be very relevant and cost effective in the identification of mutagens and thus potential genotoxic carcinogens. Since, in general, the introduction of new genotoxic substances is not accepted in the (human) environment, the detection of such properties usually prohibits further industrial development of a substance and further animal testing. Only in cases where the substance is considered very important and beneficial, will further testing be undertaken in order to determine if the substance is indeed a carcinogen and, if so, to what extent a certain human exposure poses a risk.

In vivo genotoxicity is, at times, advocated after *in vitro* testing and prior to long-term animal testing. If these tests are negative the chance of the genotoxic substance being an animal, and thus also probably a human carcinogen is reduced.

Similarly, a positive outcome of an *in vivo* genotoxicity test may prevent, in specific cases, any further testing.

In vitro genotoxicity testing usually involves at least two or preferably three of the following different tests: to detect gene mutations in a prokaryote, or in a eukaryote, a test to detect DNA damage (e.g., unscheduled DNA synthesis (UDS)) a test to detect adduct formation such as the ^{32}P-postlabelling assay of Randerath, described in 1989, and a test to detect chromosomal damage. There should be at least one test directed to chromosomal damage in a eukaryotic organism. The test in a prokaryote usually involves bacteria such as *Salmonella typhimurium* (Ames test) or *Escherichia coli* where reverse mutations are scored as an indication of genotoxic potential (OECD GLs No. 471, No. 472). The principle of this test is the detection of reverse mutations of a strain of bacteria which are growth dependent and where the reverse mutation leads to independent growth which can be detected on a feeding layer that is devoid of the growth factor. These tests are well standardized and validated.

Over 200 discrete *in vitro* genotoxicity tests have been described, but most of them are insufficiently developed and validated to be used. As a eukaryotic system, yeast cells (OECD GL No. 480) or, preferably, somatic cells are used (OECD GL No. 476). A system using mammalian cells *in vitro* where UDS is measured as an indication of genotoxicity (OECD GL No. 482) may also be used. For chromosome aberrations, the *in vitro* mammalian cytogenetic test (OECD GL No. 473) or the *in vitro* sister chromatid exchange test (SCE) in mammalian cells (OECD GL No. 479) are used. Whereas the principle of detection in eukaryotic system is in fact the same as for prokaryotic systems, the principle for detection of unscheduled DNA synthesis is derived from the ability of mammalian cells to repair damaged DNA to a certain extent and to determine such unscheduled DNA-repair by autoradiographic methods, by incorporation of tritiated thymidine. Unscheduled DNA synthesis can be differentiated from "scheduled" DNA synthesis because normal cell duplication leads to heavily labelled cells easily distinguishable from cells showing unscheduled repair, which are only lightly labelled by autoradiographically detected silver grains.

Finally, chromosome aberration tests detect structural losses or changes in the chromosomes, which can be studied by arresting the cells in mitosis and by quantification of abnormal chromosomes or exchange of chromatids in a statistically sufficient number of mitoses or cells.

The genotoxicity of a substance is usually considered to be established when at least two of three tests are positive. When all three tests are negative, there is adequate evidence that the substance has no genotoxic properties. When genotoxicity is detected it is advisable, before conducting an extensive and costly long term bioassay, to perform *in vivo* genotoxicity tests such as the mouse bone marrow micronucleus test (OECD GL No. 474), the *in vivo* cytogenetic test (OECD GL No. 475), the rodent liver genotoxicity test (not yet included in the OECD guidelines) or the rodent dominant lethal test (OECD GL No. 478). When these tests are negative, the likelihood of the substance being non-genotoxic in rodents is high (provided that there is an indication that the compound reached the target tissue) and, conversely, positive responses may make it very likely that the substance will be a genotoxic rodent carcinogen.

In the mouse bone marrow micronucleus test, micronuclei, derived from substance treated mice, are counted in a statistically sufficient number of bone marrow cells and compared to those from control mice. Without general signs of bone marrow toxicity and valid pharmacokinetic data on the test substance a negative micronucleus test is difficult to interpret.

A suitable rodent liver genotoxicity test described by Williams (1980) is the test where rats are treated with the substance of concern and where the liver cells in primary culture are submitted to tritiated thymidine exposure in order to detect increased (unscheduled) DNA-repair.

Another genotoxicity test which can be applied to an organ is the alkaline elution method described by Brunborg et al. (1988) which detects single strand DNA breaks.

In the dominant lethal test a serial mating technique is used where substance-treated males are mated with single virgin females for one oestrus cycle. By replacing one virgin female for another this breeding study is continued for 70 days, which is long enough to cover all stages of spermatogenesis. The detection of early embryonic deaths in the females is an indication of dominant lethality. In addition, this type of test also provides limited information on fertility.

In vitro cell transformation assays are generally based on carcinogen-induced loss of contact inhibition resulting in a piling up of transformed cells in disorganized criss-cross fashion. They are considered highly relevant for the process of *in vivo* carcinogenesis. They involve the same end-point, since transformed cells are tumorigenic in appropriate hosts, and very probably have many of the same cellular and molecular mechanisms as *in vivo.* Cell transformation tests have demonstrated high sensitivity for both animal and human carcinogens, and are sensitive to both genotoxic and non-genotoxic carcinogens. The overall performance of cell transformation tests in predicting human carcinogens is better than that of the *Salmonella* mutagenicity test. Although there are several types of cell transformation assays, there seems to be a generally good agreement in test results among different assays. The cell transformation assays are less widely used than the various genotoxicity assays, primarily because the former are more resource intensive and require considerable experience for proper execution and interpretation.

Genotoxicity tests provide the possibility of detecting possible genotoxicity *in vitro* and/or *in vivo,* the latter are considered to be more relevant for risk assessment because they demonstrate the potential for genetic toxicity in the presence of toxicokinetic, metabolic and cytoprotective processes. Some authorities have assumed that non-genotoxic carcinogens operate through mechanisms which have biological thresholds which is a rational for regulatory decision making to adopt a NOAEL/uncertainty factor approach. This may not be true for all such carcinogens nor is this approach universally adopted.

3.1.11 Reproductive and development toxicity

The recognition of a reproductive hazard is of great importance as there is considerable and increasing public concern about the potential effects of chemicals on

the developing embryo and fetus. Reproductive toxicity includes adverse effects on fertility in males and females and on developmental toxicity, and covers any effect interfering with normal development both before and after birth, from conception to sexual maturity. Although this definition seems sufficient and clear, it should be realized that a clear distinction between reproductive and other signs of toxicity is not always practicable or feasible. Reproductive toxicity is not synonymous to teratology (irreversible structural or functional malformations), but teratogenicity (embryo/fetotoxicity) is a part of reproductive toxicity. Although all stages of the reproductive cycle can be vulnerable to directly or indirectly induced effects, more rapid developmental changes may be more vulnerable than others.

Conception, the *development period* from conception to sexual maturity, the *embryonic period* from conception to the end of major organogenesis, the *fetal period* from the end of embryogenesis to the birth of the progeny, and the *neonatal period*, may each be involved in chemical toxicity. Not infrequently, there is a delay between the moment of exposure and the manifestation of an effect and this is especially the case in gamete formation and maturation. Gametogenesis occurs very early in embryogenesis, but germ cell formation in females occurs only before birth. Certain adverse effects in cells can be induced before birth, but are not expressed before the germ cell is fertilized and undergoes the developmental period until sexual maturity, thus bypassing one generation.

Two generations of animals with one litter per generation are generally utilized as the minimum reproduction study. The most sensitive animal species is theoretically often employed, based on the assumption that for the majority of known toxicants humans are as sensitive or more sensitive than the most sensitive animal species.

Adverse effects on reproduction can be detected by animal experimentation. Basically, reproductive toxicity can be detected during each stage of development. The selection of species is particularly important in the case of reproductive toxicity because there may be wide differences in sensitivity as evidenced by the lack of teratogenicity of thalidomide in rats. It is generally considered essential that a second non-rodent species be studied especially in relation to testing for possible teratogenicity. The rabbit is the most commonly used second species and the treatment is usually only over the period of organogenesis with assessment of effects just preparturition.

Examples of effects are given in Table 8.

Detection of reproductive toxicity in animal experiments is usually done in four segments:

1. Fertility and general reproductive performance
2. Embryotoxicity and teratogenicity
3. Pre- and post-natal development
4. Multi-generation studies

Table 8. Types of adverse effects detected in reproductive toxicity

Time and targets at which a substance initiates its toxicity	Examples for adverse effects on:
Adult toxicity	- libido - behaviour - endocrine function - mating - gamete production - reproductive life span - interaction with other hormone mediated functions

Maternal toxicity (changing physiology and metabolism during pregnancy and lactation)	- susceptibility
	- ability to nurse
	  - milk quality/quantity
	  - behaviour

Developmental toxicity	

Preimplantation and implantation	- fertilization
	  - movement of fertilized ova
	  - implantation
	  - survival of ova

Embryonic development	- growth and differentiation
	  - organ development
	  - survival

Placental development	- growth - organ function

Fetal development	- growth and differentiation
	  - function of organ system(s)
	  - survival

Postnatal development (neonatal, preweaning, postweaning, puberty)	- birth-weight
	  - organ system(s)
	  - hormone function
	  - immune function
	  - CNS and peripheral NS function
	  - sexual function
	  - other cellular functions (transplacental carcinogenesis)
	  - survival

1. Fertility and general reproductive performance involves the treatment of females and males before mating for a duration sufficiently long to cover the different stages of spermatogenesis or follicular development. Pregnancy, lactation, and development to sexual maturity are followed and recorded in comparison to controls.

2. Embryotoxicity and teratogenicity is investigated by treating pregnant mammals (usually rats and or rabbits) during embryogenesis. Fetuses are recovered just before delivery and examined for morphological and structural malformations.

3. In pre- and post-natal development studies the treatment of pregnant mammals is restricted to the last third of pregnancy and during parturition and lactation in order to examine adverse effects during that particular period.

4. For multi-general studies a simple two-generation reproduction toxicity test (OECD GL No. 416) provides an excellent and cost-effective way of testing which can provide evidence that all reproductive functions are normal. The test does not discriminate the origin of the adverse effects; this is good for detection, but usually provides poor characterization of effects.

Reproductive toxicity testing is usually carried out in phases, in order to investigate potential effects effectively and efficiently. A well known screening assay is the Chernoff/Kavlock assay developed in 1982 which is also used in the OECD priority setting of High Production Volume Chemicals with a modification where the dosing period of the animals is extended for 1 or 2 weeks prior to mating and through mating and gestation (OECD, No. 421/422). Obviously, such a screening assay offers the possibility of detecting obvious hazard, but does not offer a high assurance of absence of hazard.

When assumed exposure to chemicals is high, it is advisable to perform studies covering all the phases of the reproductive cycle, especially since in such segment studies exposure can be relatively high for a short period of time. As in other toxicity studies doses should have a low increment of 2 to a maximum of 5 between doses and should include a NOAEL as well as an obvious effect level for maternal toxicity. Typically, 20 litters per dose are used. Since the mother is the treated entity, the litter is the experimental unit. Statistical analysis should be performed on either data and the sample size is the number of litters. Embryotoxicity or teratogenicity occurring only at maternally toxic dose levels have less relevance than similar effects produced by doses that have no adverse effects on the dams.

Moreover, all available information on dynamics and kinetics should always be used in order to design the most appropriate test to obtain meaningful and reliable information. *In vitro* studies for reproductive toxicity testing are available but as yet insufficiently validated to be used routinely.

Many countries and institutions now require where relevant, test methods using animals which demonstrate the toxic effects of chemicals on reproduction.

3.1.12 Immunotoxicity studies

Although in a well performed toxicity study all types of effects may usually be detected, the focus of the study is not directed *per se* to effects on, for example, the immune system, or the central nervous system and, in particular, related behaviour.

Immunotoxicology has received growing interest in the last decade, since it is recognized that chemicals may influence the immune system in a variety of ways and affect immune responsiveness and thus maintenance of health. For example, adverse effects on the immune system could render the host more susceptible to infections and a compromised immuno-surveillance could increase susceptibility to cancer. There are two basic types of interaction of chemicals with the immune system, these are antigenic activity and immunotoxic activity.

There is considerable debate regarding the most appropriate methods for the identification of potential immunotoxicants within the context of routine toxicological investigations as well as how best to determine the relevance for man of immunomodulatory effects. One difficulty in reaching a consensus on appropriate approaches to hazard identification has been the multitude of end-points for immunological investigations. These have included: induced changes in the weight, composition and histopathological appearance of lymphoid tissues, haematological parameters and functional assays that seek to measure the activity of various components of the immune system. A tiered testing approach has been generally advocated. It has been proposed that a first tier is incorporated into the OECD Test Guideline No. 407 - Repeated dose toxicity test. In the tiered testing approach the objective of the first tier is to identify potential immunotoxicity by including specific parameters such as complete blood cell count and differential count of white blood cells, organ weights of thymus, lymph nodes, spleen and histopathological examination of thymus, spleen, lymph nodes, Peyer's patches and bronchus associated lymphoid tissue (BALT). In addition, the measurement of serum, IgM, IgG and IgA concentrations is suggested. When indications of immunotoxicity are found, further testing in specific test systems are employed to identify the immunotoxic properties and to detect the lowest level at which any effect will occur (i.e., cell mediated immunity, humoral immunity, macrophage function, natural killer function or host resistance). First tier parameters could also be added to OECD Test Guideline No. 408 - chronic toxicity testing. Finally tests of immune function also include tests of resistance to infectious challenge and host-graft rejection.

A variety of indicators of possible immunotoxicity can also be derived from animal short-term and subchronic toxicity and developmental toxicity studies which include haematology, clinical, histopathology, organ and body weight as well as morbidity and mortality indicators. Additional expanded immunotoxicity tests *per se* could include additional haematology and serum chemistry and histopathology tests as well as *in vivo* and *in vitro* analysis of the functional capacity of specific cell types.

3.1.13 Neurotoxicity studies

Neurotoxicity is defined as xenobiotic induced, biologically significant undesirable changes in the structure or function of the nervous system. These changes can be permanent or reversible and can be expressed as neuropathology or as altered neurochemical, electrophysiological or behavioral function. The perceived risks for learning disabilities and other types of neurological dysfunction in children and older adults from exposure to environmental chemicals are of serious public concern.

Neurotoxicity caused by environmental toxicants results in a broad range of neurological and behavioral disorders which can occur after long latent periods. Neurotoxicity is of practical relevance since neurotoxic effects are not infrequently found; in 24% of the chemicals for which occupational exposure limits have been set, neurotoxic effects were the only reason, or reasons in part, for regulation. Neurotoxicological signs such as neuromotor dysfunction, narcoses or seizures can be detected in the classical toxicity tests, although it is essential that the observations are conducted by experienced personnel because subtle and barely recognizable signs may be indicative of neurotoxicity. The reliable detection and interpretation of behavioral changes can be difficult because intoxicated animals may exhibit behavioral changes which are not really related to neurotoxicity. (Until recently neurotoxicity was based largely on the basis of clinical observations by trained animal facility staff). Nevertheless, neurobehavioral changes are important in assessing neurotoxicity although it must be recognized that many of the tests suggested in behavioral toxicology require validation.

A tiered approach had been proposed by Rice (1990) for studying neurobehavioral toxicity. After obtaining indications for neurotoxicity in the primary (classical) testing a more detailed analysis should be carried out. Such testing usually consists of the examination of learning and memory, and may also include evaluation of sensory and motor function. Finally, a third level of testing is suggested to characterize in detail the nature of the toxic effect and to determine the lowest level at which any effect is observed.

Neurotoxicity assays which evaluate toxicant-induced changes utilizing both *in vivo* and *in vitro* procedures and integrating data derived from behavioral, neurological, neurophysiological, neuropathological and neurochemical studies have been extensively described including those by the US FDA in 1993 and by the NRC in 1992. Neurotoxic effects on complex integrative functions such as motor performance, sensory acuity, memory and cognitive effects can only be detected *in vivo*. While there are functional observational batteries (FOBs) and tests designed to detect and measure major overt neurotoxic effects, it was considered by WHO in 1986 and NRC in 1992 that no existing validated system satisfies all the necessary requirements for a screening program to detect the neurotoxic potential of chemicals.

3.1.14 Future developments

Increasing recognition of the costs of assessing the potential health effects of thousands of substances per year coupled with increasing concern about the ethics of animal experimentation has served to catalyze efforts leading to the possible replacement or reduction in the use of animals, and the refinement of test methods to minimize the stress and suffering to animals. It has been estimated that currently the cost of testing a single substance through the whole battery of tests using whole animals is frequently in excess of 2 million dollars in the USA. Alternatives to whole-animal testing include: end-point assays, cell and tissue cultures, the use of tissue slices, toxicokinetic modeling and structure-activity relationships and database. The main questions concerning the use of alternatives are: 1) how do we extrapolate the use of *in-vitro* system to an *in-vivo* system? 2) how do we use available *in-vitro* data to design

better experimental approaches and 3) how do we predict potential biological effects from chemical-structure of a substance? Isolated cells, tissues and organs can be prepared and maintained in culture by methods that preserve the properties and characteristics of the same cells, tissues and organs *in vivo*. Particular attention has been given in the EC to validation programmes for *in vitro* tests on eye irritation and skin in relation with the European Council Directive (86/609/EEC) for the protection of animals used for experimental purposes. Several *in vitro* tests for eye irritation are being validated and a suitable alternative to the Draize eye irritation test can be expected. *In vitro* tests are being developed for dermal irritation potential and these also require validation as described by Gad & Chengelis (1988).

In regard to alternative approaches for the assessment of reproductive toxicity (with emphasis on embryotoxicity/teratogenicity), a number of *in vitro* systems which use established cell lines, primary cultures of embryonic cells, organ cultures, intact rodent embryos and free living embryos of non-mammalian species are currently in the exploratory phase and attempts have been made by Kimmel et al. (1982) and Gelbke (1993) to validate the suitability of a number of *in vitro* screening tests for teratogenic activity. The value of new tests for reproductive toxicity will depend on their ability to detect and quantify the potential for effects on reproduction. Since most new tests measure a relatively smaller spectrum of reproductive end-points than the conventional *in vivo* reproductive tests, a minimum of *in vitro* tests would be required to cover all aspects of reproduction. Only a relatively few *in vitro* assays have been adequately validated for a broad spectrum of chemicals but many are sufficiently validated to be used in certain circumstances to select materials for further testing.

A number of *in vivo* tests, examining acute toxicity, neurotoxicity, immunotoxicity, respiratory sensitization are in various stages of elaboration by the application of additional test parameters in order to augment or simplify existing procedures such as that of van der Venne & Berlin (1993). As in the case of newly developed *in vitro* tests, validation is the major difficulty to which is added problems related to classification and acceptance criteria for national and international bodies. Validation resources can include a chemical bank, cell and tissue banks, a data bank and reference laboratories as well as the formation of a scientific advisory board representing the academic, industrial and regulatory communities.

It has been stressed in many quarters that test validation process should be highly flexible and adaptive to the specific test method and proposed use. Because tests can be used for many different purposes by different types of organizations and with varying categories of substances, the determination of whether a procedure is considered to be scientifically validated must be made on a case-by-case basis in the context of the proposed use(s) of the test.

In vitro tumour promotion studies are being developed because of the high expenditure on whole animal studies. These include cell transformation assays (initiation and promotion and cell communication (promotion). With regard to carcinogenicity studies, increasing efforts are in progress, particularly in the USA to further refine dietary restriction in terms of the design and interpretation of toxicity and carcinogenicity

assays. For example, *ad libitum* feeding during long term rodent bioassays has been associated with obesity, increased incidence of spontaneous tumours and diminished life expectancy. While these changes may be reversed or eliminated if the rodents are subjected to dietary/caloric restriction, other qualitative or quantitative changes may occur which can potentially affect the animals' response to test substances. Such changes may have implications on the sensitivity of the NTP bioassay, and profound implications for subsequent risk and safety analysis.

3.2 *In vitro* studies (excluding genotoxicity)

The replacement of whole animal tests by *in vitro* tests has been a subject of debate for many years. Although not always acknowledged, *in vitro* genotoxicity testing (see Section 3.1.10) is one of the successful applications of *in vitro* tests in toxicity testing. Not necessarily as a true alternative, but merely as a pre-screen to decide if further testing is necessary rather than as a true alternative. In the last decade, *in vitro* tests have been proposed as an alternative or a pre-screen for, among others, prenatal toxicity, eye irritation, dermal irritation, tumour promotion and target organ toxicity. *In vitro* tests for acute toxicity using cell systems or unicellular organisms have also been suggested as alternatives. However, most of the tests mentioned above are not yet sufficiently validated and validation procedures, especially in the past, have been lacking in consistency. Moreover, the validation focus is usually on the need to show reproducibility and high correlation with an existing animal test, which it is intended to replace. A potentially valuable aspect of alternatives is their use in studying certain phenomena, when mechanistic similarity can be proved. For example, new end-points can be incorporated in screening programs when they may provide added insight into the mechanism of toxicity as described by Balls et al. (1992), and Seibert et al. (1994). *In vitro* tests may ultimately provide additional information to facilitate hazard and risk assessment. Recently, transgenic cell systems have been developed. Such systems may sometimes be relevant to certain aspects or end-points in toxicity and will facilitate *in vitro - in vivo* extrapolation to a considerable extent. The European Union has paid particular attention to validation programmes for *in vitro* tests for skin and eye irritation in relation to the EC Directive 86/609/EEC for the protection of animals used for experimental purposes. Table 9 depicts a scenario that could be followed to develop and validate an alternative method successfully in order to obtain international regulatory acceptance. Most importantly, the focus should be on simple discrete end-points which can be used for hazard identification. Complex processes, usually already difficult to understand *in vivo*, cannot be transposed to *in vitro* studies, but the understanding of crucial biological processes, their critical events and ultimate end-points will certainly stimulate the development of *in vitro* tests which may provide more insight at the molecular level and facilitate extrapolation across species. However, it is not possible to carry out risk assessment using *in vitro* data only.

3.2.1 Prenatal toxicity

In prenatal toxicity a large number of experimental methods are currently in different exploratory phases, using all lines, primary cultures of embryonic cells, isolated embryos, limb buds, and hydra. Fairly good correlations with *in vivo* systems

Table 9. Approaches in the development of *in vitro* test methods that might lead to regulatory acceptance (Koëter, 1993)

Step	Requirements
Scientific justification	1. Select simple endpoints essential for hazard identification 2. Develop *in vitro* assays for these end-points 3. Understand the mechanism of the *in vitro* assay and demonstrate similarities to the target event 4. Publish the assay(s) in a high quality peer-reviewed journal
Database development	5. Conduct the *in vitro* assays parallel to the relevant *in vivo* studies 6. Conduct and report all studies fully in compliance in GLP 7. Integrate results of the *in vitro* assays in dossiers submitted to regulatory agencies
International acceptance	8. Propose the *in vitro* assays to the OECD to be considered for Test Guideline Development

have been obtained in chick embryos. Systems using primary cells from embryos to study developmental processes, growth, and cell differentiation seem promising predictors of developmental toxicity, but unfortunately they mostly require the use of more animals and are labour intensive. All these systems bypass the placental barrier which in the *in vivo* situations is an important barrier limiting or even preventing contact between a number of substances and the fetus. For example, substances such as neurotoxic amino acids may give positive results whereas in reality they will not reach fetal tissues *in vivo*. The best validated systems are the *Drosophila* neuroblast-myoblast assay, mouse and rat limb bud cell assay, CNS cells in micromass culture, chick embryo neural retina cells in culture, chick embryo neural crest and limb bud cells in culture, mouse ovarian tumour cell assay, human embryonic mesenchyme assay, pox virus proliferation assay, and the neuroblastoma cell differentiation assay.

3.2.2 *Eye and skin irritancy*

For eye irritancy the "Eyetex" assay, enucleated bovine eyes and chicken egg chorioallantoic assay are proposed as worthwhile *in vitro* tests. "Eyetex", a physico-chemical assay based on protein aggregation to mimic the production of corneal opacity is well validated for a number of chemicals and has an acceptable sensitivity (91%), specificity (89%) and predictive value (93%) as compared to *in vivo* Draize results.

Enucleated bovine eyes (BE) for the prediction of corneal damage, and chicken egg chorioallantoic membranes (CAM) to estimate irritancy potential, also show a reasonable correlation with *in vivo* data (5% clear disagreement).

In vitro tests are being developed for dermal irritation potential and require requisite validation. Similarly to the "Eyetex" test for eye irritancy, the "Skintex" assay has been developed for skin irritancy, which measures reactivity spectrophotometrically in a protein matrix and shows a reasonable correlation (87%) with the *in vivo* Draize rabbit skin test. For skin irritancy the use of cellular systems in mono- and multilayers, or suspension cultures of primary epidermal cells and/or cell lines has been proposed, but these are not sufficiently validated. Assays with viable or non-viable (cadaver) skin have proved to be of only limited use, though the non-viable skin system seems promising in the identification of corrosive substances.

3.2.3 Tumour promotion

In vitro tumour promotion assays have been suggested but are as yet insufficiently validated. Most of these assays are based on the loss of cell-to-cell communication as a possible predictor of tumour promoting activity, although it has been established that powerful promoters do not necessarily affect intercellular communication. Assays for gap-junction intercellular communication have failed to respond to several chemicals that are known to be active as promoters or carcinogens *in vivo,* such as TCDD, aflatoxins and asbestos. The possibility of mechanisms of promoter action other than perturbation of intercellular communication has been advanced by Yamasaki (1990). Outgrowth of partially transformed cells is another system which is claimed to predict promoting activity.

3.2.4 Organ toxicity

Many *in vitro* methods using tissue- and primary cell cultures or cell lines are being used to provide mechanistic information on target organ toxicity. However, these tests are usually used as to investigate mechanisms as an addition to generated *in vivo* data rather than providing well validated alternatives for *in vivo* testing. An increasingly used system is primary liver cells (see 3.1.10), and cells of other organs such as thyroid, intestine, and central nervous system (astrocytes) have also been suggested.

3.3 Human studies

Evaluation of the effects of chemicals on human health is a complex process that begins with the hazard identification understood as the determination of substances of concern, their adverse effects, target populations, and conditions of exposure. Human studies provide direct insight into the adverse effects of contact with environmental agents. Their results not only complement the empirical assessments or experimental findings but are also used to validate the results of indirect hazard identification methods. An advantage of direct methods offered by human studies is that they do not involve extrapolation of data obtained in non-human experiments. However, in most cases, sufficient data are unavailable from human studies, and human studies can obviously be conducted on those substances to which substantial human exposure has already occurred. Thus they are less useful in the early identification of hazard, and they are usually irrelevant to the pre-market evaluation of new chemicals.

The database for direct human hazard identification and safety assurance arises in most instances from observational studies, especially from epidemiological investigations. In addition, information derived from case reports may be of value. In several countries, data are also generated from human volunteer studies and such data may be used in risk assessment. Human volunteer studies must incorporate ethical considerations so that unacceptable exposure situations are avoided.

All human studies should be designed, conducted and reported in accordance with four basic ethical principles, namely "respect for persons", "beneficence", "non-maleficence", and "justice". Embodied in the Declaration of Helsinki, these essential provisions for protecting human subjects form a sound basis for both observational and experimental studies. The spectrum of ethical concerns that must be addressed is wide and includes the issues of informed consent, maximizing benefit, minimizing harm, confidentiality and conflict of interest. For investigators it may at times be difficult to ensure that all of the requirements are met, particularly that new research methodologies and technologies are expanding the horizons for both epidemiological and experimental studies. In order to resolve possible ethical objections proposals for human studies should undergo independent ethical review procedures.

3.3.1 *Epidemiological studies in hazard identification*

Epidemiology, as a study of the distribution and determinants of health-related states or events in populations and the application of this study to control of health problems, exerts, by definition, an important role in every step of the process leading to the control of environmental hazards to human health. The epidemiological contribution to this process is already apparent at the stage of hazard identification. Unlike indirect means of assessing health hazards the epidemiological methods involve direct observations of human populations and deal with real life exposures and those adverse health effects that are of primary concern.

The answer to the central question of hazard identification, namely "does the agent cause the adverse health effect?" addresses the issue of causation. Epidemiological approach to this question takes into account inherent limitations of methodology and multifactorial aetiology of health disorders. In practice, the causal inference involves the evaluation of observed associations between the exposure and defined biological response, against criteria formulated on the assumption that certain features of the association are more likely than not to occur by chance. The application of practical guidelines can be traced back to the original Henle-Koch postulates and those currently used are based on the criteria first formulated by Hill (1965). The list includes strength of the association, consistency of the association, specificity of the association, temporal relationship of the association, biological gradient or dose-response curve, plausibility, coherence, experiment and analogy. The updated approach to causal inference extends the Hill's guidelines by introducing gains of recent advances in biostatistics and reflecting an ongoing debate on causative concepts in epidemiology. The number and variety of criteria illustrates well the degree of uncertainty concerning judgement of causality and "what they can do, with greater or less strength, is to help us to make up our minds on the fundamental question - is there any other way of explaining the set of

facts before us, is there any other answer equally, or more, likely than the cause and effect".

Careful interpretation of the results of epidemiological studies is advised in the light of known limitations to epidemiological inference. Epidemiological studies have often employed imperfect measures of exposure based on surrogate indices rather than on quantitative data. Quantitative exposure information is difficult to obtain and even if direct environmental measurements are available their results are subject to measurement error and usually do not cover the whole relevant period of exposure. Another source of uncertainty is difference in life-style factors and in susceptibility of the study subjects. A number of factors can also affect the validity of health outcome measurements even if standardized tools are used. Specific problems in epidemiology relate to the selection of appropriate comparison group and to the recognition of all potentially important confounding factors. Finally, statistical considerations point to the importance of sufficient size of the study group required for statistically valid inference. With this caveat in mind it is important that the results of epidemiological studies are viewed with caution. As a rule each epidemiological report requires special consideration of the above determinants of internal and external validity in epidemiological research, i.e., the degree to which the epidemiological inference is warranted. Internal validity is satisfied when the observed differences between the study and comparison groups may, apart from sampling error, be attributed only to the hypothesized effect under investigation. External validity refers to the requirement that the study results produce inferences that can be generalized with regard to a specified external target population. Well conducted epidemiological studies have succeeded in documenting a role of environmental exposures among causative factors of diseases, particularly in occupationally exposed populations.

It should be noted that epidemiological studies with negative results cannot prove the absence of intrinsic hazardous properties of a substance. However, well documented negative studies of good quality are useful in the risk assessment. An example can be furnished by studies that showed no significant health risk related to the use of saccharine. Anecdotal information showing no effects is of very little value as long as the circumstances have not been adequately recorded.

3.3.1.1 Design of epidemiological studies

The main observational strategies in epidemiology to estimate prevalence or incidence of health events are cross-sectional design, cohort or longitudinal design and case-control design. Prevalence is the proportion of individuals who have a given health event at a specific time calculated as the number of individuals with the health event at a specified point of time divided by the size of the population at that time. Incidence refers to the number of health events or other attributes that occur in a given period and is calculated as the frequency of the occurrence of new health events per observation period relative to the size of the population.

A number of epidemiological strategies have been applied to hazard identification. However, given the type of information sought in hazard identification, the basic design

options are most frequently used to identify environmental determinants of health disorders.

Cross-sectional studies involve concurrent assessment of health status and exposure status, although historical information on exposure can be also available. This type of studies examine the prevalence, not incidence, of health events such as specified health disorders or other defined health attributes. The results of comparison of prevalence between the study groups defined according to the presence or absence of exposure are used to demonstrate exposure-specific occurrence pattern of examined health events. Statistical relationships between exposure variables and response variables (health events) can be analyzed by measures of association such as prevalence difference, prevalence ratio or odds ratio. Cross-sectional studies are particularly useful for generating aetiological hypotheses regarding environmental exposures that can be studied by other methods.

Cohort studies involve longitudinal observation of subjects whose exposure is known at the beginning of the observational period. Depending on the chronological direction in which study subjects are followed cohort studies can be divided into prospective, retrospective or ambispective designs. (Retrospective studies are more common since suspicions of the presence of a chemical hazard are usually associated with risk management strategies to minimize any possible adverse health effects).

Definition of a cohort according to the presence or absence of exposure factor of interest and subsequent follow-up allows measurement of the incidence rate of the examined health outcome. Causal inference is based on the results of incidence rates comparison between exposed and unexposed cohorts. Data from cohort studies can be used to assess association between the exposure of interest and the occurrence of the examined health events. The commonly used measures of association include standardized morbidity or mortality ratios of the corresponding differences in incidence rates. The cohort design is the preferred method for studying environmental determinants of health disorders. However, this type of study usually involves long follow-up periods and is less suitable for studying rare health outcomes.

Case-control study is a valuable and relatively inexpensive tool to study etiologic hypotheses of rare diseases. This study design involves assessment of past exposure of subjects with the examined health outcome ("case subjects") and the comparison subjects who do not have the health outcome of interest ("control subjects"). The difference in distribution of the examined exposure factor between cases and controls suggests possible causal links. A variant is the nested case-control study in which cases and control subjects are in a cohort study. This modification may be considered as a cost-effective way to analyze the data arising from longitudinal observation. The major advantage of case-control design is that it provides good estimates of relative risk but without the full processing of data from all subjects who do not develop the disease. Within a cohort study population or within a geographical population, exposure information has to be obtained and processed for all diseased subjects and for a random population sample. The measure of association is the odds ratio, calculated as the product of numbers of exposed cases and unexposed controls divided by the product of

numbers of exposed controls and unexposed cases. This measure is an estimate of relative risk describing exposure-health event association. Appropriate selection of controls is a main difficulty with the design. Furthermore, bias may be introduced when exposure data are obtained retrospectively, for example by interview or questionnaire at the time when disease has occurred in the cases.

For each of the above methods, it is essential that there are reliable exposure data in the study populations and that the extents of exposure differ sufficiently to allow the study to detect any relationship.

3.3.1.2 Measurement of exposure and health effects in epidemiology

The key principle of epidemiological investigation into identification of a hazardous agent is that the population distribution of exposure to the agent is associated with the population distribution of health effect. Accordingly, appropriate measurements of exposure and health effects are central to epidemiological approach to hazard identification. Inaccuracies in either regard will tend to produce type II errors, i.e., failure to demonstrate an effect when one exists.

In environmental epidemiology assessment of exposure to hazardous agents is a difficult and challenging task. It involves not only the measurements of concentrations of substances of interest in environmental media and the measurement of the duration of exposure but also should respond to interindividual differences in absorption and disposition of specific substances as they are found and distributed within the environment. The lack of sufficiently sensitive and selective analytical methodologies for many compounds and chemical mixtures have frequently led to subsequent difficulties in epidemiological assessment of exposure (e.g. mixtures of aromatic hydrocarbons, amines, metals).

Depending on the study protocol the exposure to a hazardous agent can be analyzed on individual or group level and should take into account various sectors of environment, including the domestic environment, the occupational environment, the local (or community) environment and the regional environment. Total exposure to a chemical is a function of its concentrations in all relevant environments and media, frequency and duration of contacts between a chemical and exposed person. The assessment of exposure to environmental agents is further discussed in Section 5.3.

In environmental epidemiology a desired approach is the measurement of internal exposure (absorbed dose) that provides a more relevant assessment of actual human exposure than external exposure measurement. The US EPA Total Exposure Assessment Methodology (TEAM) Studies (as delineated by Wallace, 1993) showed that exposures estimated from the measurement of outdoor and indoor concentrations of chemicals underestimated individual exposures by large margins, suggesting that individual activities contribute very much to actual exposure. However, even the best available assessment of internal exposure to chemicals is always confronted by the questions of interindividual variation in body burden and target dose, as well as individual variation in susceptibility to chemical toxicity, due to differences in toxicokinetic patterns.

The measurement of individual exposure is expensive and limited to those agents for which personal sampling techniques or validated quantitative biomarkers of exposure have been developed. Commonly, epidemiological inferences are based on exposure data provided by less perfect measures. Exposure information is usually available at a population or group level. If health effects are also recorded at an aggregate (population, group) level the association found between exposure and health effects may not reflect the association that exists at an individual level ("ecological fallacy").

Adverse health effects of environmental exposure to chemicals include a large spectrum of subjective or objective biological responses, ranging from subject discomfort to death. Because of the variety of potential responses (end-points) characterizing chemical toxicity the central issue in the epidemiological evaluation of health effect is its definition. Depending on the study objective, individual health outcome can be classified according to standard definitions or using a study-specific definition. For ultimate end-points, such as deaths, information provided by mortality statistics offices is the best available source of data, despite doubts about the causes of deaths recorded on the of death certificates. International classification of causes of deaths provides standards for mortality studies. Morbidity can be evaluated based on disease registers, individual hospital or out-patient records. However, morbidity data collected through study-specific direct examination can be more suitable for individual health assessment, particularly as existing records are subject to potential diagnostic variation. With current diagnostic techniques, the definition of health outcome can reflect different levels of biological response, ranging from overt clinical entities to subclinical changes, including isolated functional or structural alterations at the molecular level.

In general, epidemiological evaluation of health status is concerned with two broad categories: categorical and continuous measures of health events. The choice of the measure of examined health effect implies its presentation on group/population level for risk estimation. Categorical end-points are measured as prevalence or incidence rates. To describe continuous end-points the measures of central tendency or distribution, such as mean, standard deviation, mode and median, are used.

The commonly used indices of risk involving comparisons of the population occurrence of health effects include absolute risk, relative risk, odds ratio, standardized mortality ratio, proportionate mortality ratio and attributable risk. When health effects are expressed as quantitative end-points the relation between continuous variables can be examined by methods for assessing associations, such as for example correlation analysis or analysis of variance. Both categories of health effect data can be analyzed using modelling techniques as described by Greenland (1989). Multivariate modelling of epidemiological data, useful in analysis of multifactorial aetiology diseases, should follow a sound model of suspected impact of environmental exposure on human health, and respond to the nature of relationship between examined health effect and exposure.

The majority of data that have been obtained in the past deals with human health effects linked with acute or chronic exposure to single agents. Much work is needed to

describe the effects of exposures to mixtures of environmental chemicals. The goal is challenging and fully justified by the magnitude of major health problems affecting human populations. Known as diseases of multifactorial aetiology they include cancer, cardiovascular and pulmonary diseases, immunotoxicological disturbances, reproductive abnormalities and neurobehavioral disorders.

In human studies the availability and quality of pathological data is important. Depending on the end-points, histopathological confirmation may be essential in confirming effects that have structural and morphological components. Furthermore, refined tools to examine functional alterations resulting from environmental exposure are needed to widen the range of adverse health effects studied in epidemiology. In this area, a close integration between animal and human studies is necessary to improve definition of exposure-specific changes that might be used for better hazard identification and assessment of exposure-response relationships.

3.3.1.3 Biomarkers of exposure, susceptibility and effect in epidemiological studies

Recognized imperfect measures of exposures and adverse health effects in human studies have led increasingly to the introduction of biomarkers into epidemiological studies. Biomarkers linked with toxic effects of exposure to chemicals are classified as biomarkers of exposure, biomarkers of effect and biomarkers of susceptibility. The following definitions by WHO (1993), specify each category:

> "Biomarker of exposure is defined as an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism.
>
> Biomarker of effect is defined as measurable biochemical, physiological, behavioral or other alteration within an organism that, depending upon the magnitude, can be recognized as associated with an established or possible health impairment or disease.
>
> Biomarker of susceptibility is defined as indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance".

There is an overlap between biomarkers of exposure and biomarkers of effect because the same biomarkers can be used for both measurements. Some of the same biomarkers are also used to measure interindividual differences in response and thus in a further sense are biomarkers of susceptibility. Table 10 lists a number of biological exposure markers. There is a continuum between biological markers of exposure and those of effect (Fig. 9). Biomarkers can be divided into those that measure compartments, e.g., blood levels, DNA adducts, or tissue damage and those that measure mechanisms, e.g., enzyme activity levels or gene polymorphisms. (Each has its advantages and limitations). Fig. 10 illustrates the conceptual basis for the development of biomarkers for use in molecular epidemiology; SCE, sister chromatid exchange. Fig. 10a depicts the development of biologically based risk assessment models. Data

Table 10. Examples of biological markers

Agent	Exposure marker
Aniline	Total *p*-aminophenol in urine; methaemoglobin in blood
Benzene	Total phenol or muconic acid in urine; benzene in expired air
Cadmium	Cadmium in urine or blood
Carbon disulfide	2-Thiothiazolidine-4-carboxylic acid in urine; CO in end-expired air
Chlorobenzene	Total 4-chlorocatechol or *p*-chlorophenol in urine
Chromium VI	Total Cr in urine
N,N-Dimethylformamide	*N*-Methylformamide in urine
Ethylbenzene	Mandelic acid in urine or ethylbenzene in end-expired air
Fluorides	Urinary fluoride
Furfural	Total furoic acid in urine
n-Hexane	2,5-Hexanedione in urine or *n*-hexane in expired air
Lead	Lead in blood or urine, ZPP
Methanol	Methanol or formic acid in urine
Methaemoglobin inducers	Methaemoglobin
Methyl chloroform	Methyl chloroform in end-expired air or trichloroacetic acid in blood or urine
Methyl ethyl ketone	Urinary methyl ethyl ketone
Nitrobenzene	Total *p*-nitrophenol in urine or methaemoglobin in blood
Organophosphates	Red cell or serum cholinesterase activity
Parathion	Total *p*-nitrophenol in urine or RBC cholinesterase
Pentachlorophenol (PCP)	Total PCP in urine or free PCP in plasma
Styrene	Mandelic acid in urine, styrene in blood; phenylglyoxylic acid in urine
Toluene	Hippuric acid in urine; toluene in blood or end-expired air
Trichloroethylene (TCE)	Trichloracetic acid in urine; TCA or trichloroethanol in blood; TCE in end-expired air
Xylenes	Methylhippuric acids in urine

from multiple sources are used to construct models, knowledge gaps are identified which when filled lead to refined models with less uncertainty. Fig. 10b is an illustrative scheme of molecular epidemiology using benzo(a)pyrene (BP) as a model (as suggested by Perera in 1996).

For individuals or populations at risk, it is preferable to have measurements both from mechanistic avenues and from related compartments to more effectively target prevention strategies. Fig. 11 illustrates a continuum illustrating how polymorphisms in environmental response genes can modify an individual's risk for disease.

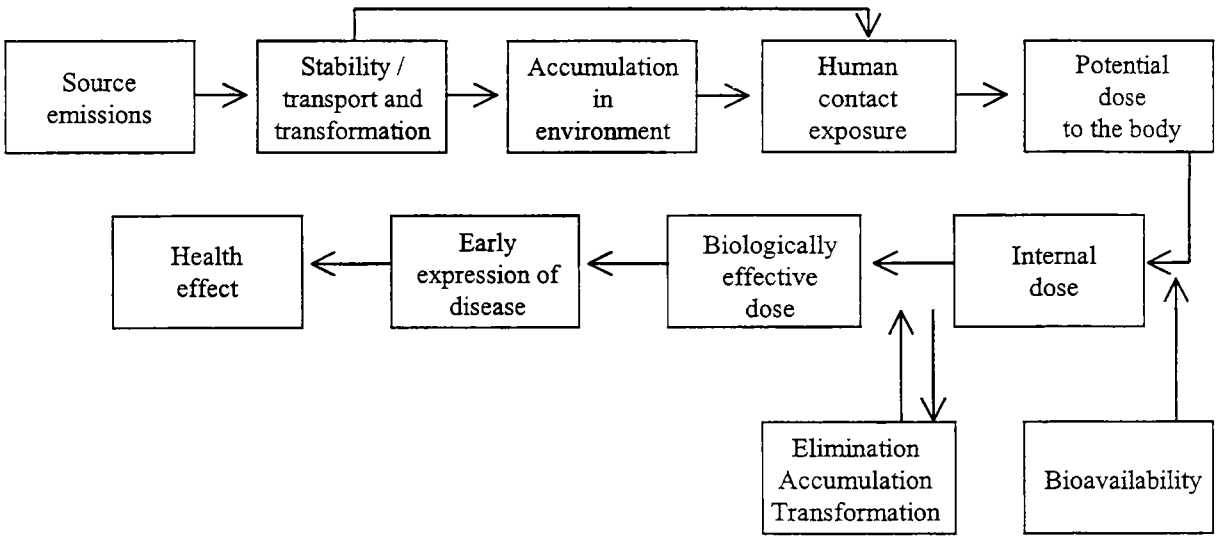

Fig. 9. Continuum from emission to a health effect

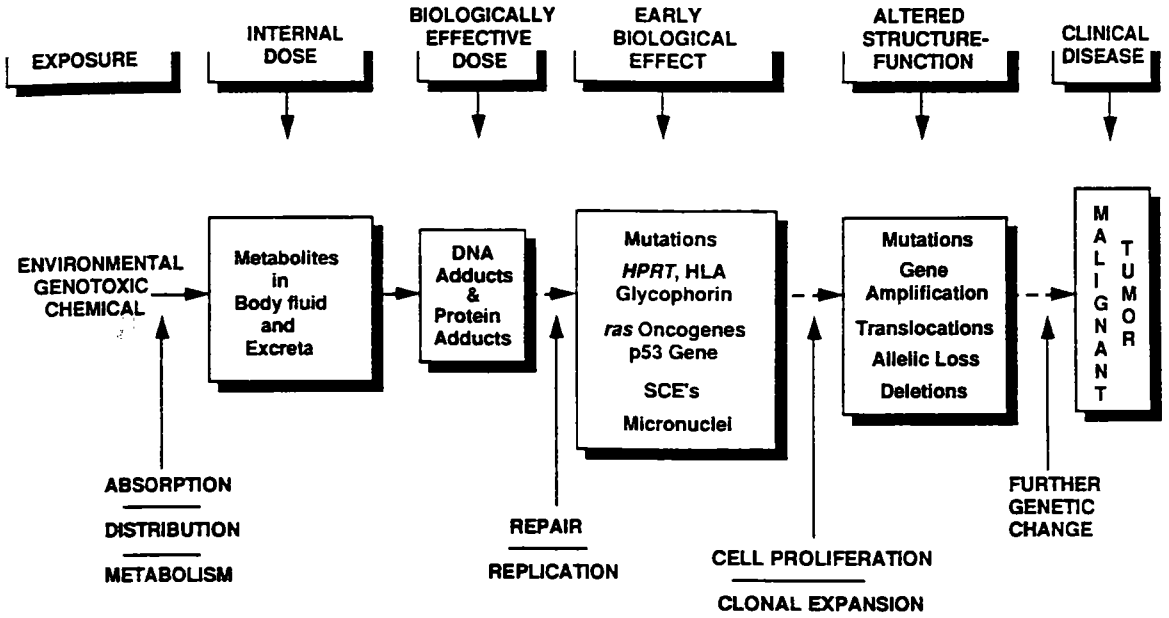

Fig. 10. Conceptual basis for the development of biomarkers for use in molecular epidemiology

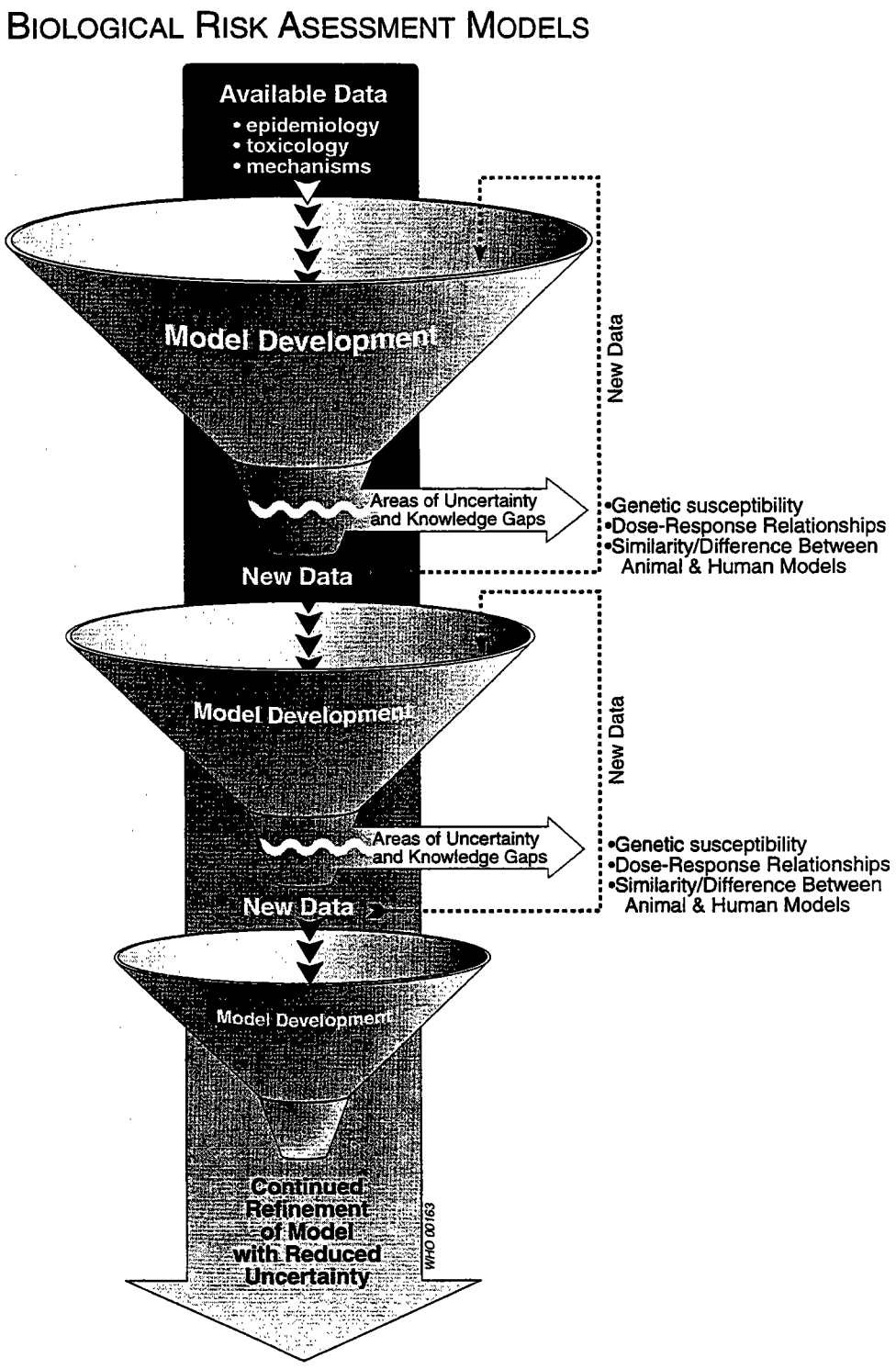

Fig. 10a. Development of biologically based risk assessment models

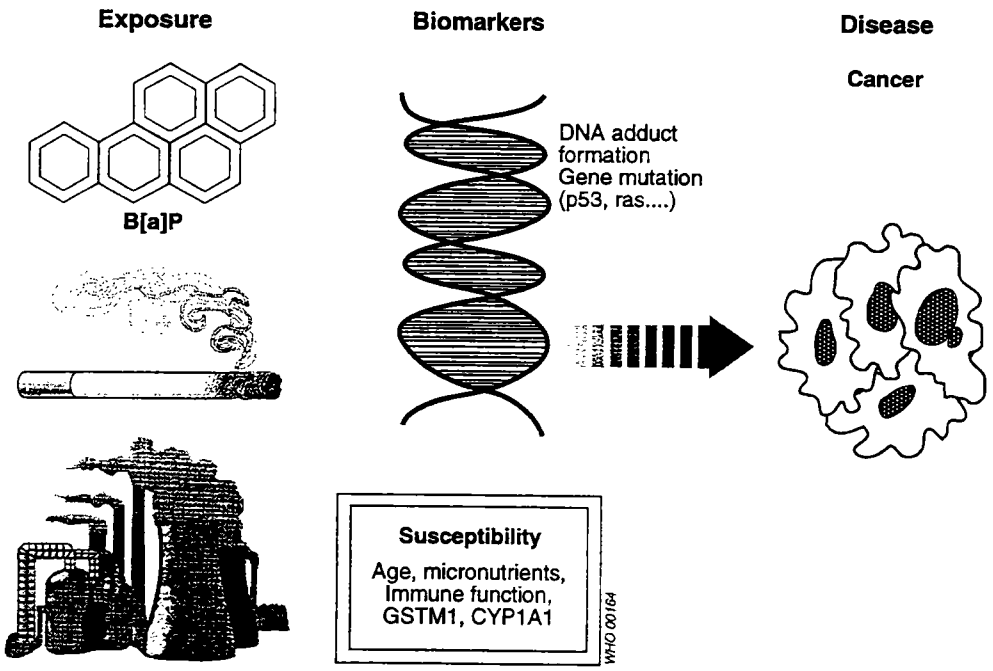

Fig. 10b. Illustrative scheme of molecular epidemiology using benzo(a) pyrene (BP) as a model

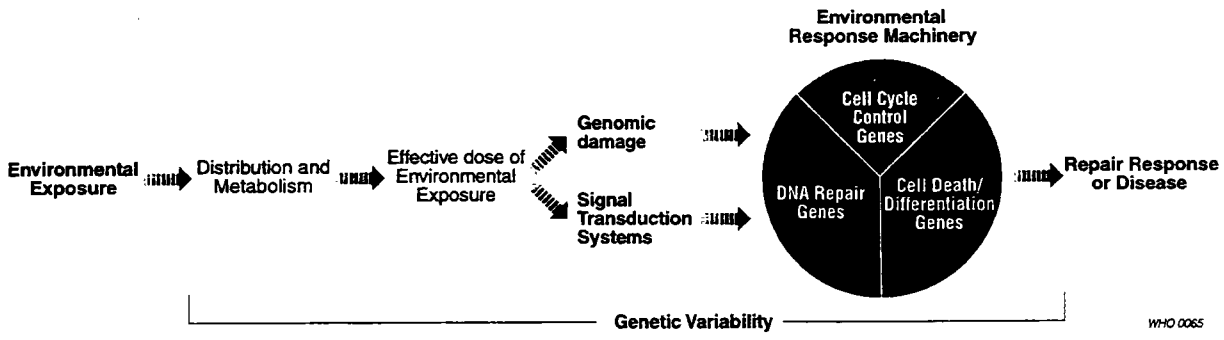

Fig. 11. Polymorphisms in environmental response genes

Analysis of biomarkers is increasingly being incorporated into cross-sectional, retrospective or nested case-control studies to gain improved resolution of the risk factors and mechanisms responsible for cancer. The introduction of biomarkers has enhanced epidemiological research but at the same time has posed a number of problems related to validation of biomarker methods, their reliability, practical value, as well as ethical implications. Current developments in the field of molecular biological investigations have provided more specific techniques to measure biological responses to exposures. As a result, epidemiological studies using biomarkers can provide more detailed information on toxic effects of environmental factors. A promising application of biomarkers is their use as "molecular dosimeters". In cancer epidemiology the list includes covalent DNA adducts or protein adducts and their excretion products. However, these biomarkers differ in terms of their availability, reliability, sensitivity and specificity, and hence their validity should be established if used in risk assessment. Even with the advent of well validated techniques molecular epidemiology will remain subject to inherent problems of human studies that deal with a complex issue of interplay between the host, the environment, and the agent.

3.3.1.4 Quality of epidemiological information

Reliable hazard identification in epidemiological studies depends on the precise formulation of study objectives, preparation of objectives-specific study protocol, choice of unequivocal definitions of exposure and health effects, recognition of potential confounding and modifying factors, appropriate choice of study subjects, accuracy of data obtained during implementation phase and their appropriate analysis and interpretation.

Among numerous factors affecting the quality of epidemiological information there are two well recognized sources of uncertainty, referred to as random error and systematic error. Random error refers to the lack of precision in estimating study parameters and usually reflects imperfect accuracy of measurements of both exposure and biological response under study. The presence of random error affects the power of the study and when investigated association is weak this type of error may result in failure to demonstrate its existence. Random error cannot be entirely avoided but it can be reduced by adherence to appropriate principles of data collection and analysis.

Systematic error is a potent source of distortion of epidemiological information. This type of error refers to the lack of internal validity of the study. Known also as bias, it is responsible for the study results that depart systematically from the true value. Bias can operate at any stage of epidemiological investigation and can be categorized into selection bias, information bias and confounding bias.

Quality of epidemiological reports is an important issue in a growing body of scientific literature. The published data may however provide an incomplete picture. Partly it may be due to an editorial predilection for publishing positive findings, partly because authors may prefer to submit reports with positive findings. This factor, known as bias in publication has to be taken into account when a review of existing evidence is attempted. A conclusive synthesis of published evidence on the same problem can be facilitated by statistical methods for combined analysis or meta-analysis.

Meta analysis, or the analysis of analyses, is a technique first developed in the social sciences in the 1980s that provides a method for combining results across studies without having access to the data from these studies. While meta analysis allows the qualitative incorporation of more data into risk assessment, selection of such data must be toxicologically valid.

The principal reasons for conducting a meta-analysis (or a review) are to: 1) assess qualitatively whether a factor has to be considered a risk factor; 2) to provide more precise effect estimate and to increase the statistical power and to analyze dose-response relations; 3) to investigate the heterogeneity between different studies; 4) to generalize results of single studies; 5) to investigate rare exposures and interactions and 6) investigate risks associated with rare diseases. For example, meta analyses have been principally used to access weak risk factors that have a large public health impact such as passive smoking, the exposure to low-level ionizing radiation, exposure to electromagnetic fields or to indoor radon.

The use of meta analysis remains, to date, controversial since many investigators believe that meta analysis from published data are in general insufficient to calculate a pooled estimate since published estimates are often based on heterogenous populations, different study designs and mainly different statistical models.

3.3.2 Case reports in hazard identification

In several important instances, the clinical observation of a few cases of intoxication has suggested an aetiological role of environmental or occupational exposures; e.g., as in the cases of MPTP, hexachlorobenzene, dibromochloropropane, and methyl ethyl butyl ketone which were all recognized. An astute clinician is an important element in hazard identification. Usually, in clinical setting, it is not easy to recognize the nature and source of the incriminated exposure, especially since attending physician may not be sufficiently trained in environmental health. Another factor that affects the evaluation of cases suspected for chemical-induced disease is individual variation in susceptibility to chemical toxicity, apparent in epidemiological studies and much more an issue in clinical practice. The picture may be less complicated when a sudden outbreak of a disease occurs in a cluster and all or majority of cases are treated in one health centre. In general, local health effects of exposure to chemicals are more evident than systemic effects, and similar distinction can me made between diagnostic features of acute and chronic health effects.

In general, case reports can offer the first clues of environmental factors playing a role in a disease outbreak. However, their most important disadvantages are that they lack statistical reliability and do not allow for control of important confounders, and thus cannot be used to establish causal associations.

3.3.3 Human volunteer studies

Human volunteer studies have been permitted and performed in various countries following appropriate investigations in animals. Human experimentation should be

limited to those chemicals and those circumstances of administration that are proven to be safe by animal or other studies. These studies can increase the reliability of risk assessment in toxicology. The examples include controlled human exposures to air pollutants in chambers that enable examination of specific physiologic and cellular responses and human tolerance studies in food toxicology. Their relevance arises from the fact that subjects are free from significant health disorder that might have affected the results of experiments and that the conditions of experiment are under control. Furthermore human studies provide insight into symptoms and/or health effects that cannot be studied in animal experiments or can hardly be studied in epidemiological studies. Because of the conditions of scientific experiment human volunteer studies are well suited to examine the risk of exposure to environmental agents according to the paradigm of exposure - dose - response. With the use of adequate research tools this type of study provides opportunities to evaluate both toxicokinetics and toxicodynamics in human models. It is important to realize that the results of volunteer studies cannot substitute wholly for the results of non-human toxicological test programmes. At a present time their strength stems from the fact that in number of instances effects observable in humans cannot be readily reproduced '*in vitro*' or in animal experiments (e.g. idiosyncratic human responses).

In applying the experimental method to humans it is necessary to observe the rule of a "minimal risk", in accordance with two complementary ethical principles for the conduct of research on human subjects, namely non-maleficence and beneficence. In any case, the potential benefits of experiments must be carefully weighted against the probable risk, and the adverse effects must be carefully monitored and controlled. If healthy volunteers are not exposed to unacceptable risk and ethical requirements are fulfilled the risk of participation in non-therapeutic research may be no greater than that of everyday life. Furthermore, beneficence toward humans in biomedical experiments requires that all human volunteer studies are performed according to the Guidelines of Good Clinical Practice (EC, 1990). These guidelines provide ethical, scientific and technical rules that are crucial to guarantee the safety of subjects and the quality of test results in volunteer studies.

It should be emphasized that human volunteer studies should never be performed automatically as part of toxicological test programmes. In principle, studies in healthy volunteers should only be considered when they add knowledge that cannot be obtained in animal or *in vitro* experiments.