Toxicology in Occupational and Environmental Setting

Toxicology

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Abbreviations:

Ah-R	arylhydrocarbon receptor
AP	apurinic/apyrimidinic site
APS	adenosine 5′-phosphosulfate
BHK	baby hamster kidney
BIBRA	British Industrial Biological Research Association
CoA	Coenzym A
DDT	1,1′-(2,2,2-trichloroethylidene)bis-(4-chlorobenzene)
DHHS	U.S. Department of Health and Human Services
DHP	delayed hypersensitive response
ECETOC	European Chemical Industry Ecology and Toxicology Centre
ED	effective dose
ELISA	enzyme-linked immunosorbent assay
FCA	Freund's complete adjuvant
FAD	flavine adenine dinucleotide
GABA	γ-aminobutyrate
GC/MS	gas chromatography/mass spectroscopy
GOT	glutamic acid oxalacetic transaminase
GSH	glutathione
GSSG	glutathione disulfide
GST	glutathione *S*-transferase
GTP	guanosine 5′-triophosphate
HGPRT	hypoxanthine – guanine phosphoribosyltransferase
IPCS	International Programme on Chemical Safety
LDH	lactate dehydrogenase
LOAEL	lowest-observed-adverse-effect level
LOEL	lowest-observed-effect level
MIF	migration inhibition factor

mRNA	messenger RNA
MTD	maximum tolerated dose
NADPH	nicotinamide dinucleotide phosphate (H)
NOEL	no-observed-effect-level
NTP	National Toxicology Program
PAPS	3′-phosphoadenosine-5′-phosphosulfate
PG	prostaglandin
rRNA	ribosomal RNA
SHE	Syrian hamster embryo
SMART	somatic mutation and recombination test
T, or TCDD	2,3,7,8-tetrachlorodibenzodioxin
TD	tumor dose
TK	thymidine kinase
tRNA	transfer RNA
UDP	uridine diphosphate
UDPG	uridine diphosphate glucose
UDPGA	uridine diphosphate glucuronic acid
UDS	unscheduled DNA synthesis

1. Introduction

1.1. Definition and Scope

Chemicals that are used or of potential use in commerce, the home, the environment, and medical practice may present various types of harmful effects. The nature of these effects is determined by the physicochemical characteristics of the agent, its ability to interact with biological systems (hazard), and its potential to come into contact with biological systems (exposure).

Toxicology studies the interaction between chemicals and biological systems to determine the potential of chemicals to produce adverse effects in living organisms. Toxicology also investigates the nature, incidence, mechanisms of production, factors influencing their development, and reversibility of such adverse effects. Adverse effects are defined as detrimental to the survival or the normal functioning of the individual. Inherent in this definition are the following key issues in toxicology:

1) Chemicals must come into close structural and/or functional contact with tissues or organs to cause injury.
2) All adverse effects depend on the amount of chemical in contact with the biological system (the dose) and the inherent toxicity of the chemical (hazard). When possible, the observed toxic effect should be related to the degree of exposure. The influence of different exposure doses on the magnitude and incidence of the toxic effect should be quantitated. Such dose-response relationships are of prime importance in confirming a causal relationship between chemical exposure and toxic effect (for details, see Section 1.7).

Research in toxicology is mainly concerned with determining the potential for adverse effects caused by chemicals, both natural and synthetic, to assess their hazard and risk of human exposure and thus provide a basis for appropriate precautionary, protective and restrictive measures. Toxicological investigations should permit evaluation of the following characteristics of toxicity:

1) The basic structural, functional, or biochemical injury produced
2) Dose-response relationships
3) The mechanisms of toxicity (fundamental biochemical alterations responsible for the induction and maintenance of the toxic response) and reversibility of the toxic effect
4) Factors that modify response, e.g., route of exposure, species, and gender

For chemicals to which humans may potentially be exposed, a critical analysis, based on the pattern of potential exposure or toxicity, may be necessary in order to determine the risk-benefit ratio for their use in specific circumstances and to devise protective and precautionary measures. Indeed, with drugs, pesticides, food additives, and cosmetic preparations, toxicology testing must be performed in accordance with government regulations before use.

1.2. Fields

Toxicology is a recognized scientific discipline encompassing both basic and applied issues. Although only generally accepted as a specific scientific field during this century, its principles have been appreciated for centuries. The harmful or lethal effects of certain chemicals, mainly present in minerals and plants or transmitted

venomous animals, have been known since prehistoric times. In many countries, toxicology as a discipline has developed from pharmacology. Pharmacology and toxicology both study the effect of chemicals on living organisms and have often used identical methods. However, fundamental differences have developed. Years ago, only the dependence on dose of the studied effects separated pharmacology and toxicology. Pharmacology focused on chemicals with beneficial effects (drugs) at lower doses whereas toxicology studied the adverse health effects occurring with the same chemicals at high doses. Today, the main interest of research in toxicology has shifted to studies on the long-term effects of chemicals after low-dose exposure, such as cancer or other irreversible diseases; moreover, most chemicals of interest to toxicologists are not used as drugs.

The variety of potential adverse effects and the diversity of chemicals present in our environment combine to make toxicology a very broad science. Toxicology uses basic knowledge from clinical and theoretical medicine and natural sciences such as biology and chemistry (Fig. 1). Because of this diversity, toxicologists usually specialize in certain areas.

Any attempt to define the scope of toxicology must take into account that the various subdisciplines are not mutually exclusive and frequently are heavily interdependent. Due to the overlapping mechanisms of toxicity, chemical classes, and observed toxic effects, clear divisions into subjects of equal importance are often not possible.

The professional activities of toxicologists can be divided into three main categories: descriptive, mechanistic, and regulatory. The *descriptive toxicologist* is concerned directly with toxicity testing. Descriptive toxicology still often relies on the tools of pathology and clinical chemistry, but since the 1970s more mechanism-based test systems have been included in toxicity testing [1]. The appropriate toxicity tests in experimental animals yield information that is extrapolated to evaluate the risk posed by exposure to specific chemicals. The concern may be limited to effects on humans (drugs, industrial chemicals in the workplace, or food additives) or may encompass animals, plants, and other factors that might disturb the balance of the ecosystem (industrial chemicals, pesticides, environmental pollutants).

The *mechanistic toxicologist* is concerned with elucidating the mechanisms by which chemicals exert their toxic effects on living organisms. Such studies may result in the development of sensitive predictive toxicity tests useful in obtaining information for risk assessment (see Chap. 4). Mechanistic studies may help in the development of chemicals that are safer to use or of more rational therapies for intoxications. In addition, an understanding of the mechanisms of toxic action also contributes to the knowledge of basic mechanisms in physiology, pharmacology, cell biology, and biochemistry. Indeed, toxic chemicals have been used with great success as mechanistic tools to elucidate mechanisms of physiological regulation. Mechanistic toxicologists are often active in universities; however, industry and government institutions are now undertaking more and more research in mechanistic toxicology.

Regulatory toxicologists have the responsibility of deciding on the basis of data provided by the descriptive toxicologist and the mechanistic toxicologist if a drug or chemical poses a sufficiently low risk to be used for a stated purpose. Regulatory toxicologists are often active in government institutions and are involved in the establishment of standards for the amount of chemicals permitted in ambient air in the environment, in the workplace, or in drinking water. Other divisions of toxicology may be based on the classes of chemicals dealt with or application of knowledge from toxicology for a specific field (Table 1).

Forensic toxicology comprises both analytical chemistry and fundamental toxicologic principles. It is concerned with the legal aspects of the harmful effects of chemicals on humans. The expertise of the forensic toxicologist is invoked primarily to aid in establishing the cause of death and elucidating its circumstances in a postmortem investigation. The field of *clinical toxicology* recognizes and treats poisoning, both chronic and acute. Efforts are directed at treating patients poisoned by chemicals and at the development of new techniques to treat these intoxications. *Environmental toxicology* is a relatively new area that studies the effects of chemicals released by man on wildlife and the ecosystem and thus indirectly on human health.

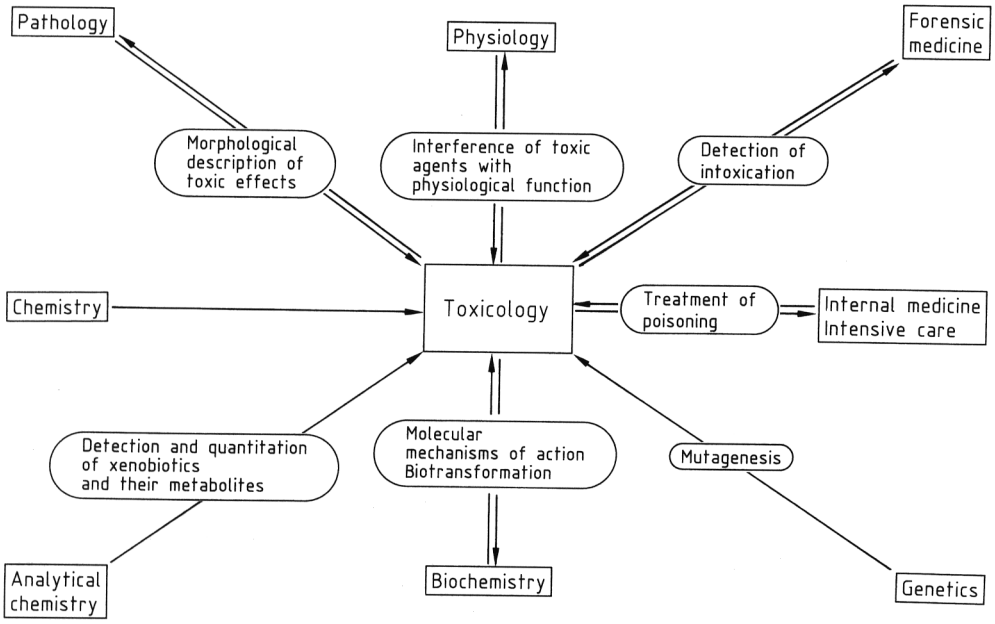


Figure 1. Scientific fields influencing the science of toxicology

Table 1. Areas of toxicology

Field	Tasks and objectives
Forensic toxicology	diagnoses poisoning by analytical procedures
Pesticide toxicology	studies the safety of pesticides, develops new pesticides
Occupational toxicology	assesses potential adverse effects of chemicals used in the workplace, recommends protective procedures
Drug toxicology	studies potential effects of drugs after high doses, elucidates mechanisms of sideeffects
Regulatory toxicology	develops and interprets toxicity testing programs and is involved in controlling the use of chemicals
Environmental toxicology	studies the effects of chemicals on ecosystems and on humans after low-dose exposure from the environment

Drug toxicology plays a major role in the preclinical safety assessment of chemicals intended for use as drugs. Drug toxicology also elucidates the mechanisms of side effects observed during clinical application. *Occupational toxicology* studies the acute and chronic toxicity of chemicals encountered in the occupational environment. Both acute and chronic occupational poisonings have exerted a major influence on the development of toxicology in general. Occupational toxicology also helps in the development of safety procedures to prevent intoxications in the workplace and assists in the definition of exposure limits. *Pesticide toxicology* is involved in the development of new pesticides and the safety of pesticide formulations. Pesticide toxicology also characterizes potential health risks to the general population caused by pesticide residues in food and drinking water.

1.3. History

Toxicology must rank as one of the oldest practical sciences because humans, from the very beginning, needed to avoid the numerous toxic plants and animals in their environment. The presence of toxic agents in animals and plants was known to the Egyptian and Greek civilisations. The papyrus Ebers, an Egyptian papyrus dating from about 1 500 B.C., and the surviving medical works of HIPPOCRATES, ARISTOTLE, and THEOPHRASTUS, published during the period 400–250 B.C., all included some mention of poisons.

The Greek and Roman civilizations knowingly used certain toxic chemicals and extracts for hunting, warfare, suicide, and murder. Up

to the Middle Ages, toxicology was restricted to the use of toxic agents for murder. Poisoning was developed to an art in medieval Italy and has remained a problem ever since, and much of the earlier impetus for the development of toxicology was primarily forensic. There appear to have been few advances in either medicine or toxicology between the time of GALEN (131–200 A.D.) and PARACELSUS (1493–1541). The latter laid the groundwork for the later development of modern toxicology. He clearly was aware of the dose–response relationship. His statement that "All substances are poisons; there is none that is not a poison. The right dose differentiates a poison and a remedy," is properly regarded as a landmark in the development of the science of toxicology. His belief in the value of experimentation also represents a break with much earlier tradition. Important developments in the 1700s include the publication of RAMAZZINI's *Diseases of Workers*, which led to his recognition as the father of occupational medicine. The correlation between the occupation of chimney sweepers and scrotal cancer by POTT in 1775 is also noteworthy.

ORFILA, a Spaniard working at the University of Paris, clearly identified toxicology as a separate science and wrote the first book devoted exclusively to it (1815). Workers of the later 1800s who produced treatises on toxicology include CHRISTISON, KOBERT, and LEWIN. They increased our knowledge of the chemistry of poisons, the treatment of poisoning, the analysis of both xenobiotics and toxicity, as well as modes of action and detoxication. A major impetus for toxicology in the 1900s was the use of chemicals for warfare. In World War I, a variety of poisonous chemicals were used in the battlefields of France. This provided stimulus for work on mechanisms of toxicity as well as medical countermeasures to poisoning. Since the 1960s, toxicology has entered a phase of rapid development and has changed from a science that was almost entirely descriptive to one in which the study of mechanisms has become the prime task. The many reasons for this include the development of new analytical methods since 1945, the emphasis on drug testing following the thalidomide tragedy, the emphasis on pesticide testing following the publication of Rachel Carson's *Silent Spring* and public concern over environmental pollution and disposal of hazardous waste.

1.4. Information Resources

Because of the complexity of toxicology as a science and the impact of toxicological investigations on legislation and commerce, a wide range of information on the toxic effects of chemicals is available. No single, exhaustive source of toxicological data exists; several sources are required to obtain comprehensive information on a particular chemical. Printed sources are often quicker and easier to use than computer data bases, but interactive online searching can rapidly gather important information from the huge number of sources present.

The information explosion in toxicology has resulted in a comprehensive volume dedicated to toxicological information sources:

P. Wexler, P. J. Hakkinen, G. Kennedy, Jr. F. W.Stoss, *Information Resources in Toxicology*, 3rd ed., Academic Press, 1999.

Textbooks. The easiest way to obtain information on general topics in toxicology and secondary references are a range of textbooks available on the market. Only a few selected books are listed below:

C. D. Klaasen, *Casarett and Doull's Toxicology; The Basic Science of Poisons*, 6th ed., McGraw-Hill, New York, 2001.

G. D. Clayton, F. E. Clayton (eds): *Patty's Industrial Hygiene and Toxicology*, Wiley, New York, 1993.

J. G. Hardman, L. E. Limbird, *Goodman and Gilman's, The Pharmacological Basis of Therapeutics*, 10th ed., McGraw-Hill, New York, 2001.

W. A. Hayes, *Principles and Methods of Toxicology*, 3rd ed., Raven Press, New York, 2001.

E. Hodgson (Ed.): *Textbook of Modern Toxicology*, 3rd ed., Wiley Interscience, 2004.

T. A. Loomis, A. W. Hayes, *Loomis's Essentials of Toxicology*, 4th ed., Academic Press, San Diego, 1996.

The huge volume by N. I. Sax and R. J. Lewis, *Dangerous Properties of Industrial Materials*, 7th ed., Wiley, New York, 1999, contains basic toxicological data on a large selection of chemicals (almost 20 000) and may serve as a useful guide to the literature for compounds not covered in other publications.

Monographs. The best summary information on toxicology is published in the form of series by governments and international organizations. Most of these series are summarizing the results of toxicity studies on specific chemicals. The selection of these chemicals is mainly based on the extent of their use in industry (e.g. trichloroethene), their occurrence as environmental contaminants (mercury) or their extraordinary toxicity (e.g. 2,3,7,8-tetrachlorodibenzodioxin):

American Conference of Governmental Industrial Hygienists, Threshold Limit Values and Biological Exposure Indices (Cincinnati, OH). Published annually.

MAK-Begründungen, VCH Publishers, Weinheim, Federal Republic of Germany. This German series includes detailed information on the toxicity of chemicals on the German MAK list (ca. 150 reports are available; the series is continuously expanded).

The Commission of the European Communities publishes the Reports of the Scientific Committee on Cosmetology and the Reports of the Scientific Committee for Food.

The Environmental Protection Agency (EPA) publishes a huge number of reports and toxicological profiles. They are indexed in "EPA Publications. A Quarterly Guide."

The European Chemical Industry Ecology and Toxicology Centre (ECETOC) issues "Monographs" (more than 20 have been published) and "Joint Assessments of Commodity Chemicals."

The monographs of the International Agency for Research on Cancer are definitive evaluations of carcinogenic hazards. The "Environmental Health Criteria" documents of the International Programme on Chemical Safety (IPCS) assess environmental and human health effects of exposure to chemicals, and biological or physical agents. A related "Health and Safety Guide" series give guidance on setting exposure limits for national chemical safety programs.

The National Institute for Occupational Safety and Health (NIOSH), has published 50 "Current Intelligence Bulletins" on health hazards of materials and processes at work.

The technical report series of the National Toxicology Program (NTP) reports results of their carcinogenicity bioassays, which include summaries of the toxicology of the chemicals studied. A status report indexes both studies that are under way and those that have been published. The program also issues an "Annual Review of Current DHHS [U.S. Department of Health and Human Services], DOE [U.S. Department of Energy] and EPA Research" related to toxicology.

A large number of internet-based resources are also available to collect information on toxic effects of chemicals and methods for risk assessment. Some information sites containing large amounts of downloadable information are listed below:

US Environmental Protection Agency (EPA), Integrated Risk Information System (IRIS), http://www.epa.gov/iris/index.html

US Environmental Protection Agency (EPA), ECOTOX Database, http://www.epa.gov/ecotox/

Organisation for Economic Co-operation and Development (OECD), test guidelines, http://www.oecd.org

Agency for Toxic Substances and Disease Registry (ATSDR), toxicological profile information sheet http://www.atsdr.cdc.gov/toxprofiles/

European Chemicals Bureau, http://ecb.jrc.it/

National Toxicology Programm, http://ntp-server.niehs.nih.gov/htdocs/liason/-Factsheets/FactsheetList.html

United Nations Environment Programm, Chemicals http://www.chem.unep.ch/

Journals Results of toxicological research are published in more than 100 journals. Those listed below mainly publish research closely related to toxicology, but articles of relevance may also be found in other biomedical journals:

Archives of Environmental Contamination and Toxicology
Archives of Toxicology
Biochemical Pharmacology
Chemical Research in Toxicology
CRC Critical Reviews in Toxicology
Clinical Toxicology
Drug and Chemical Toxicology
Environmental Toxicology and Chemistry

Table 2. Toxic effects of different chemicals categorized by time scale and general locus of action

Exposure	Site	Effect	Chemical
Acute	local	lung edema	chlorine gas
	systemic	liver damage	carbon tetrachloride
		narcosis	halothane
Subchronic	local	sensitization	toluene diisocyanate
	systemic	neurotoxicity	hexane
Chronic	local	bronchitis	sulfur dioxide
		nasal carcinoma	formaldehyde
	systemic	bladder carcinoma	4-amino-biphenyl
		kidney damage	cadmium

Food and Chemical Toxicology
Fundamental and Applied Toxicology
Journal of the American College of Toxicology
Journal of Analytical Toxicology
Journal of Applied Toxicology
Journal of Biochemical Toxicology
Journal of Toxicology and Environmental Health
Neurotoxicology and Teratology
Pharmacology and Toxicology
Practical In Vitro Toxicology
Regulatory Toxicology and Pharmacology
Reproductive Toxicology
Toxicology
Toxicology and Applied Pharmacology
Toxicology and Industrial Health
Toxicology In Vitro
Toxicology Letters

Databases and Databanks. Electronic sources, such as computer data bases or CD-ROM are a fast and convenient way to obtain references on the toxicity of chemicals. Since on-line searching of commercial data bases such as STN-International may be expensive, CD-ROM-based systems are increasingly being used. The major advantages are speed, the ability to refine searches and format the results, and non-text search options, such as chemical structure searching on Beilstein and Chemical Abstracts.

Useful information about actual research on the toxicology of chemicals may be obtained by searching Chemical Abstracts or Medline with the appropriate keywords. Specific data banks covering toxicology are the Registry of Toxic Effects of Chemical Substances, which gives summary data, statistics, and structures; Toxline (available in DIMDI) gives access to the literature.

1.5. Terminology of Toxic Effects

Toxic effects may be divided according to timescale (acute and delayed), general locus of action (local, systemic, organ specific), or basic mechanisms of toxicity (reversible versus irreversible). *Acute toxic effects* are those that occur after brief exposure to a chemical. Acute toxic effects usually develop rapidly after single or multiple administrations of a chemical; however, acute exposure may also produce *delayed toxicity*. For example, inhalation of a lethal dose of HCN causes death in less than a minute, whereas lethal doses of 2,3,7,8-tetrachlorodibenzodioxin will result in the death of experimental animals after more than two weeks. *Chronic effects* are those that appear after repetitive exposure to a substance; many compounds require several months of continuous exposure to produce adverse effects. Often, the chronic effects of chemicals are different from those seen after acute exposure (Table 2). For example, inhalation of chloroform for a short period of time may cause anesthesia; long-term inhalation of much lower chloroform concentrations causes liver damage. Carcinogenic effects of chemicals usually have a long latency period; tumors may be observed years (in rodents) or even decades (in humans) after exposure.

Toxic effects of chemicals may also be classified based on the type of interaction between the chemical and the organism. Toxic effects may be caused by reversible and irreversible interactions (Table 3). When reversible interactions are responsible for toxic effects, the concentration of the chemical present at the site of action is

the only determinant of toxic outcome. When the concentration of the xenobiotic is decreased by excretion or biotransformation, a parallel decrease of toxic effects is observed.

Table 3. Reversible and irreversible interactions of chemicals with cellular macromolecules as a basis for toxic response

Mechanism	Toxic response	Example
Irreversible inhibition of Esterase	neurotoxicity	tri-*o*-cresylphosphate
Covalent binding to DNA	cancer	dimethylnitrosamine
Reversible binding to Hemoglobin	oxygen deprivation in tissues	carbon monoxide
Cholinesterase	neurotoxicity	carbamate pesticides

After complete excretion of the toxic agent, toxic effects are reduced to zero (see below). A classical example for reversible toxic effects is carbon monoxide. Carbon monoxide binds to hemoglobin and, due to the formation of the stable hemoglobin–carbon monoxide complex, binding of oxygen is blocked. As a result of the impaired oxygen transport in blood from the lung, tissue oxygen concentrations are reduced and cells sensitive to oxygen deprivation will die. The toxic effects of carbon monoxide are directly correlated with the extent of carboxyhemoglobin in blood, the concentration of which is dependent on the inhaled concentration of carbon monoxide. After exhalation of carbon monoxide and survival of the acute intoxication, no toxic effect remains (Fig. 2).

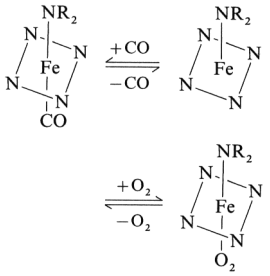


Figure 2. Reversible binding of carbon monoxide to hemoglobin and inhibition of oxygen transport

Irreversible toxic effects are often caused by the covalent binding of toxic chemicals to biological macromolecules. Under extreme conditions, the modified macromolecule is not repaired; after excretion of the toxic agent, the effect persists. Further exposure to the toxic agent will produce additive effects; many chemicals carcinogens are believed to act through irreversible changes (see Section 2.5.6).

Another distinction between types of effects may be made according to the general locus of action. *Local toxicity* occurs at the site of first contact between the biological system and the toxic agent. Local effects to the skin, the respiratory tract, or the alimentary tract may be produced by skin contact with a corrosive agent, by inhalation of irritant gases, or by ingestion of tissue-damaging materials. This type of toxic responses is usually restricted to the tissues with direct contact to the agent. However, life-threatening intoxications may occur if vital organs like the lung are damaged. For example, inhaled phosgene damages the alveoli of the lung and causes lung edema. The massive damage to the lung results in the substantial mortality observed after phosgene intoxication.

The opposite to local effects are *systemic effects*. They are characterized by the absorption of the chemical and distribution from the port of entry to a distant site where toxic effects are produced. Except for highly reactive xenobiotics, which mainly act locally, most chemicals act systemically. Many chemicals that produce systemic toxicity only cause damage to certain organs, tissues, or cell types within organs. Selective damage to certain organs or tissues by systemically distributed chemicals is termed organ- or tissue-specific toxicity [2]; the organs damaged are referred to as target organs (Table 4).

Table 4. Organ-specific toxic effects induced by chemicals that are distributed systemically in the organism

Chemical	Species	Target organ
Benzene	humans	bone marrow
Hexachlorobutadiene	rodents	damage to proximal tubules of the kidney
Paraquat	rodents, humans	lung
Tri-*o*-cresylphosphate	humans	nervous system
Cadmium	humans	kidney
1,2-Dibromo-3-chloropropane	humans, rodents	testes
Hexane	rodents, humans	nervous system
Anthracyclines	humans	heart

Major target organs for toxic effects are the central nervous system and the circulatory system followed by the blood and hematopoietic system and visceral organs such as the liver or the kidney. For some chemicals, both local and systemic effects can be demonstrated; moreover, chemicals producing marked local toxicity may also cause systemic effects as secondary responses to major disturbances in homeostasis of the organism.

1.6. Types of Toxic Effects

The spectrum of toxic effects of chemicals is broad, and their magnitude and nature depend on many factors such as the physiocochemical properties of the chemical and its toxicokinetics, the conditions of exposure, and the presence of adaptive and protective mechanisms. The latter factors include physiological mechanisms such as adaptive enzyme induction, DNA repair, and others. Toxic effects may be transient, reversible, or irrversible; some are deleterious and others are not. Toxic effects may take the form of tissue pathology, aberrant growth processes, or altered biochemical pathways. Some of the more frequently encountered types of injury constituting a toxic response are described in the following.

Immune-mediated hypersensitivity reactions by antigenic materials are toxic effects often involved in skin and lung injury by repeated contact to chemicals resulting in contact dermatitis and asthma. Inflammation is a frequently observed local response to the application of irritant chemicals or may be a component of systemic injury. This response may be acute with irritant or tissue damaging materials or chronic with repetitive exposure to irritants. Necrosis, that is, death of cells or tissues, may be the result of various pathological processes resulting from biochemical interactions of xenobiotics, as described in Chapter 3. The extent and patterns of necrosis may be different for different chemicals, even in the same organ. Chemical tumorigenesis or carcinogenesis (induction of malignant tumors) is an effect often observed after chronic application of chemicals. Due the long latency period and the poor prognosis for individuals diagnosed with cancer, studies to predict the potential tumorigenicity of chemicals have developed into a major area of toxicological research. Developmental and reproductive toxicology are concerned with adverse effects on the ability to conceive, and with adverse effects on the structural and functional integrity of the fetus. Chemicals may interfere with reproduction through direct effects on reproductive organs or indirectly by affecting their neural and endocrine control mechansims. Developmental toxicity deals with adverse effects on the conceptus through all stages of pregnancy. Damage to the fetus may result in embryo reabsorption, fetal death, or abortion. Nonlethal fetotoxicity may be expressed as delayed maturation, decreased birth weight, or structural malformation. The most sensitive period for the induction of malformation is during organogenesis; neurobehavioral malformations may be induced during later stages of pregnancy.

1.7. Dose–Response: a Fundamental Issue in Toxicology

In principle, a poison is a chemical that has an adverse effect on a living organism. However, this is not a useful definition since toxic effects are related to dose. The definition of a poison thus also involves quantitative biological aspects. At sufficiently high doses, any chemical may be toxic. The importance of dose is clearly seen with molecular oxygen or dietary metals. Oxygen at a concentration of 21% in the atmosphere is essential for life, but 100% oxygen at atmospheric pressure causes massive lung injury in rodents and often results in death. Some metals such as iron, copper, and zinc are essential nutrients. When they are present in insufficient amounts in the human diet, specific disease patterns develop, but in high doses they can cause fatal intoxications. Toxic compounds are not restricted to man-made chemicals, but also include many naturally occurring chemicals. Indeed, the agent with the highest toxicity is a natural poison found in the bacterium *Clostridium botulinum* (LD_{50} 0.01 μ/kg).

Therefore, all toxic effects are products of the amount of chemical to which the organism is exposed and the inherent toxicity of the chemical; they also depend on the sensitivity of the biological system.

The term “dose” is most frequently used to characterize the total amount of material to

which an organism is exposed; dose defines the amount of chemical given in relation to body weight. Dose is a more meaningful and comparative indicator of exposure than the term exposure itself. Dose usually implies the exposure dose, the total amount of chemical administered to an organism or incorporated into a test system. However, dose may not be directly proportional to the toxic effects since toxicity depends on the amount of chemical absorbed. Usually, dose correctly describes only the actual amount of chemical absorbed when the chemical is administered orally or by injection. Under these circumstances, the administered dose is identical to the absorbed dose; other routes of application such as dermal application or inhalation do not define the amount of agent absorbed.

Different chemicals have a wide spectrum of doses needed to induce toxic effects or death. To characterize the acute toxicity of different chemicals, LD_{50} values are frequently used as a basis for comparisons. Some LD_{50} values (rat) for a range of chemicals follow:

Ethanol	12 500
Sodium bicarbonate	4 220
Phenobarbital sodium	350
Paraquat	120
Aldrin	46
Sodium cyanide	6.4
Strychnine	5
1,2-Dibromoethane	0.4
Sodium fluoroacetate	0.2
2,3,7,8-Tetrachlorodibenzodioxin	0.01

Certain chemicals are very toxic and produce death after administration of microgram doses, while others are tolerated without serious toxicity in gram doses. The above data clearly demonstrate that the toxicity of a specific chemical is related to dose. The dependence of the toxic effects of a specific chemical on dose is termed dose–response relationship. Before dose–response relationships can be appropriately used, several basic assumptions must be considered. The first is that the response is due to the chemical administered. It is usually assumed that the responses observed were a result of the various doses of chemical administered. Under experimental conditions, the toxic response usually is correlated to the chemical administered, since both exposure and effect are well defined and can be quantified. However, it is not always apparent that the response is the result of specific chemical exposure. For example, an epidemiologic study might result in discovery of an "association" between a response (e.g., disease) and one or more variables including the estimated dose of a chemical. The true doses to which individuals have been exposed are often estimates, and the specificity of the response for that chemical is doubtful.

Further major necessary assumptions in establishing dose–response relationships are:

- A molecular site (often termed receptor) with which the chemical interacts to produce the response. Receptors are macromolecular components of tissues with which a chemical interacts and produces its characteristic effect.
- The production of a response and the degree of the response are related to the concentration of the agent at the receptor.
- The concentration of the chemical at the receptor is related to the dose administered. Since in most cases the concentration of an administered chemical at the receptor cannot be determined, the administered dose or the blood level of the chemical is used as an indicator for its concentration at the molecular site.

A further prerequisite for using the dose–response relationship is that the toxic response can be exactly measured. A great variety of criteria or end points of toxicity may be used. The ideal end point should be closely associated with the molecular events resulting from exposure to the toxin and should be readily determined. However, although many end points are quantitative and precise, they are often only indirect measures of toxicity. For example, changes in enzyme levels in the blood can be indicative of tissue damage. Patterns of alterations may provide insight into which organ or system is the site of toxic effects. These measures usually are not directly related to the mechanism of toxic action. The dose–response relationship combines the characteristic of exposure and the inherent toxicity of the chemical. Since toxic responses to a chemical are usually functions of both time and dose, in typical dose–response relationships, the maximum effect observed during the time of observation is plotted against the dose to give time-independent curves. The time-independent dose–response relationship may be

used to study dose–response for both reversible and irreversible toxic effects. However, in risk assessments that consider the induction of irreversible effects such as cancer, the time factor plays a major role and has important influences on the magnitude or likelihood of toxic responses. Thus, for this type of mechanism of toxic action, dose–time–response relationships are better descriptors of toxic effects.

The dose–response relationship is the most fundamental concept in toxicology. Indeed, an understanding of this relationship is essential for the study of toxic chemicals.

From a practical point of view, there are two different types of dose–response relationships. Dose–response relationships may be quantal (all or nothing responses such as death) or graded. The graded or variable response involves a continual change in effect with increasing dose, for example, enzyme inhibition or changes in physiological function such as heart rate. Graded responses may be determined in an individual or in simple biochemical systems. For example, addition of increasing concentrations of 2,3,7,8-tetrachlorodibenzodioxin to cultured mammalian cells results in an increase in the concentration of a specific cytochrome P450 enzyme in the cells (for details of mechanisms, see Section 2.5.4.1). The increase is clearly dose related and spans a wide range (Fig. 3). An example for a graded toxic effect in an individual may be inflammation caused by skin contact with an irritant material. Low doses cause slight irritation; as the amount increases, irritation turns to inflammation and the severity of inflammation increases.

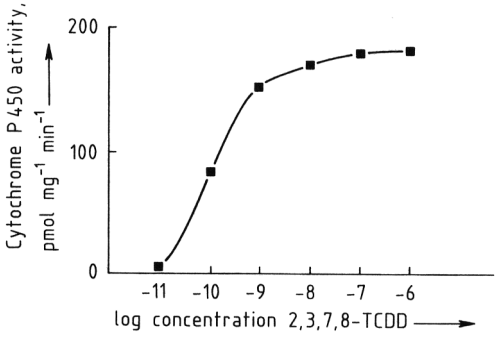


Figure 3. Dose-dependent induction of cytochrome P450 1A 1 protein in cultured liver cells treated with 2,3,7,8-tetrachlorodibenzodioxin [3]

In dose–response studies in a population, a specific endpoint is also identified and the dose required to produce this end point is determined for each individual in the population. Both dose-dependent graded effects and quantal responses (death, induction of a tumor) may be investigated. With increasing amount of a chemical given to a group of animals, the magnitude of the effect and/or the number of animals affected increase. For example, if an irritant chemical is applied to the skin, as the amount of the material increases, the numbers of animals affected and the severity of inflammation increases. Quantal responses such as death induced by a potentially lethal chemical will also be dose-dependent. The dose dependency of a quantal effect in a population is based on individual differences in the response to the toxic chemical. A specific amount of the potentially lethal xenobiotic given to a group of animals may not kill all of them, but as the amount given increases, the proportion of animals killed increases.

Althought the distinctions between graded and quantal dose–response relationships are useful, the two types of responses are conceptually identical. The ordinate in both cases is simply labeled response, which may be the degree of response in an individual, or the fraction of a population responding, and the abscissa is the range of administered doses.

1.7.1. Graphics and Calculations

Even with a genetically homogenous population of animals of the same species and strain, the proportion of animals showing the effect will increase with dose (Fig. 4A). When the number of animals responding is plotted versus the logarithm of the dose, a typical sigmoid curve with a log-normal distribution that is symmetrical about the midpoint, is obtained (Fig. 4B).

When plotted on a log-linear scale, the obtained normally distributed sigmoid curve approaches a response of 0% as the dose is decreased, and 100% as the dose is increased, but theoretically never passes through 0 or 100%. Small proportions of the population at the right- and left-hand sides of the curve represent hyposusceptible and hypersusceptible members. The slope of the dose–reponse curve around the 50% value, the midpoint, gives an indication of the

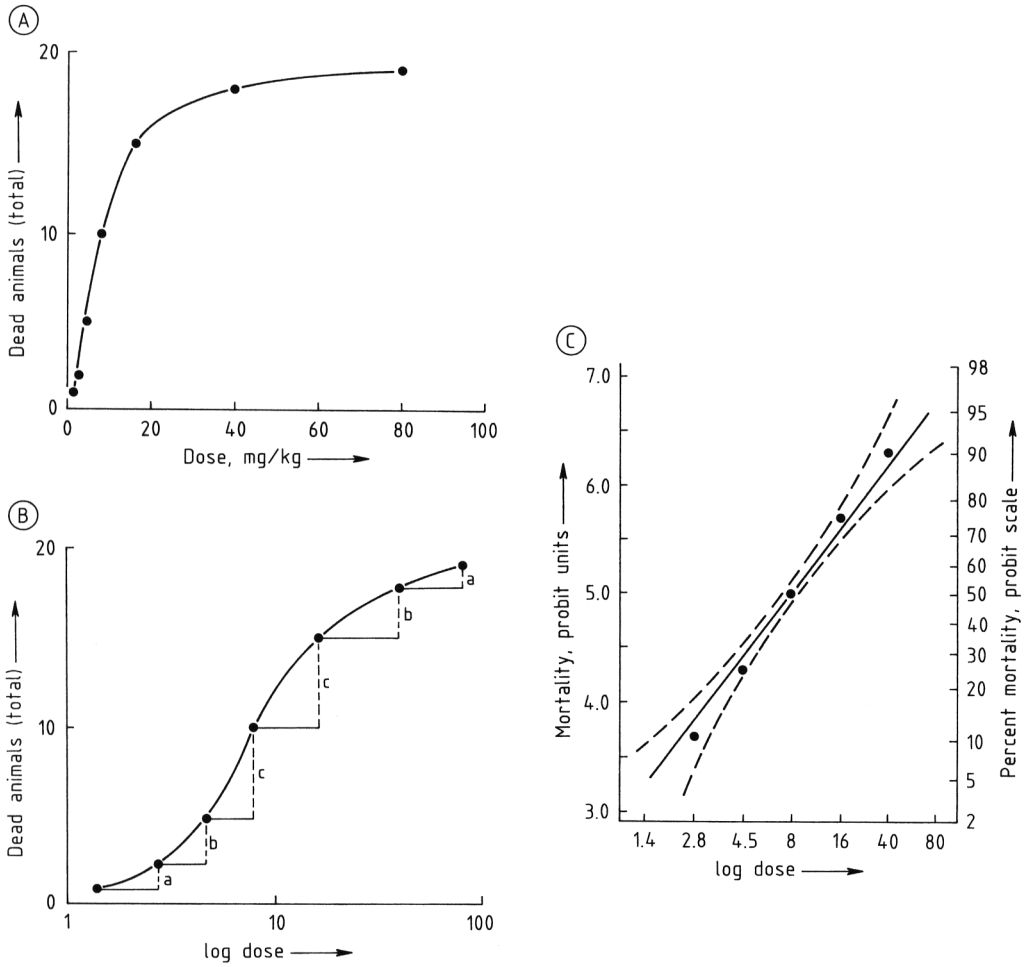


Figure 4. Typical dose – response curves for a toxic effect
Plots are linear – linear (A); log – linear (B); and log – probit (C) for an identical set of data

ranges of doses producing an effect. A steep dose–response curve indicates that the majority of the population will respond over a narrow dose range; a shallow dose–response curve indicates that a wide range of doses is required to affect the majority of the population. The curve depicted in Fig. 4B shows that the majority of the individuals respond about the midpoint of the curve. This point is a convenient description of the average response, and is referred to as the median effective dose (ED_{50}). If mortality is the endpoint, then this dose is referred as median lethal dose (LD_{50}).

Death, a quantal response, is simple to quantify and is thus an end point incorporated in many acute toxicity studies. Lethal toxicity is usually calculated initially from specific mortality levels obtained after giving different doses of a chemical; the 50% mortality level is used most frequently since it represents the midpoint of the dose range at which the majority of deaths occur. This is the dose level that causes death of half of the population dosed. The LD_{50} values are usually given in milligrams of chemical per kilogram of body weight (from the viewpoint of chemistry and for comparison of relative potencies of different chemicals, giving the LD_{50} in moles of chemical per kilogram body weight would be desirable). After inhalation, the reference is to LC_{50} (LC = lethal concentration), which, in contrast to LD_{50} values, depends on the time of exposure; thus, it is usually expressed

as X-hour LC_{50} value. The LD_{50} or LC_{50} values usually represent the initial information on the toxicity of a chemical and must be regarded as a first, but not a quantitative, hazard indicator that may be useful for comparison of the acute toxicity of different chemicals [3].

Similar dose–effect curves can, however, be constructed for cancer, liver injury, and other types of toxic responses. For the determination of LD_{50} values and for obtaining comparative information on dose–response curves, plotting log dose versus percent response is not practical since large numbers of animals are needed for obtaining interpretable data. Moreover, other important information on the toxicity of a chemical (e.g., LD_{05} and LD_{95}) cannot be accurately determined due to the slope of sigmoid curve. Therefore, the dose–response curve is transformed to a log-probit (probit = probability units) plot. The data in the Fig. 4B form a straight line when transformed into probit units (Fig. 4C). The EC_{50} or, if death is the end point, the LD_{50} is obtained by drawing a horizontal line from the probit unit 5, which is the 50% response point, to the dose–effect line. At the point of intersection a vertical line is drawn, and this line intersects the abscissa at the LD_{50} point. Information on the lethal dose for 90% or for 10% of the population can also be derived by a similar procedure. The confidence limits are narrowest at the midpoint of the line (LD_{50}) and are widest at the two extremes (LD_{05} and LD_{95}) of the dose–response curve. In addition to permitting determination of a numerical value for the LD_{50} of a chemical with few groups of dosed animals, the slope of the dose–response curve for comparison between toxic effects of different chemicals is obtained by the probit transformation [4].

The LD_{50} by itself, however, is an insufficient index of lethal toxicity, particulary if comparisons between different chemicals are to be made. For this purpose, all available dose–response information including the slope of the dose–response line should be used. Figure 5 demonstrates the dose–response curves for mortality for two chemicals.

The LD_{50} of both chemicals is the same (10 mg/kg). However, the slopes of the dose–response curves are quite different. Chemical A exhibits a "flat" dose–response curve: a large change in dose is required before a significant change in response will be observed. In contrast, chemical B exhibits a "steep" dose–response curve, that is, a relatively small change in dose will cause a large change in response. The chemical with the steep slope may affect a much larger proportion of the population by incremental increases in dose than chemicals having a shallow slope; thus, acute overdosing may be a problem affecting the majority of a population for chemicals with steeper slopes. Chemicals with shallower slopes may represent a problem for the hyperreactive groups at the left-hand side of the dose–response curve. Effects may occur at significantly lower dose levels then for hyperreactive groups exposed to chemicals with a steep dose–response.

While the LD_{50} values characterize the potential hazard of a chemical, the risk of an exposure is determined by the hazard multiplied by the exposure dose. Thus, even very toxic chemicals like the poison of *Clostridium botulinum* pose only a low risk; intoxications with this compound are rare since exposure is low. Moreover, acute intoxications with other highly toxic agents such as mercury salts are rarely seen, despite detectable blood levels of mercury salts in the general population, since the dose is also low. On the other hand, compounds with low toxicity may pose a definite health risk when doses are high, for example, constituents of diet or chemicals formed during food preparation by heat treatment.

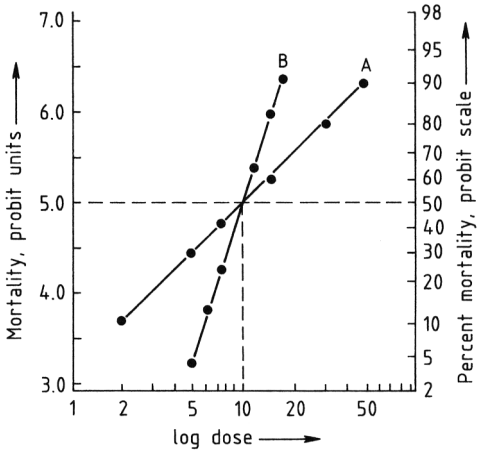


Figure 5. Comparison of dose – response relationships for two chemicals (log – probit plot)
Both chemicals have identical LD_{50} values, but different slopes of the dose – response curve

Therefore, for characterizing the toxic risk of a chemical, besides information on the toxicity, information on the conditions of exposure are necessary. When using LD_{50} values for toxicity characterisation, the limitations of LD_{50} values should be explicitly noted. These limitations include methodological pitfalls influenced by

1) Strain of animal used
2) Species of animal used
3) Route of administration
4) Animal housing

and intrinsic factors limiting the use of LD_{50} values

1) Statistical method
2) No dose–response curve
3) Time to toxic effect not determined
4) No information on chronic toxicity

The most serious limitation on the use of LD_{50} values for hazard characterization are the lack of information on chronic effects of a chemical and the lack of dose–response information. Chemicals with low acute toxicity may have carcinogenic or teratogenic effects at doses that do not induce acute toxic responses. Other limitations include insufficient information on toxic effects other than lethality, the cause of death, and the time to toxic effect. Moreover, LD_{50} values are not constant, but are influenced by many factors and may differ by almost one order of magnitude when determined in different laboratories.

1.8. Dose-Response Relationships for Cumulative Effects

After chronic exposure to a chemical, toxic response may be caused by doses not showing effects after single dosing. Chronic toxic responses are often based on accumulation of either the toxic effect or of the administered chemical. Accumulation of the administered chemical is observed when the rate of elimination of the chemical is lower than the rate of administration. Since the rate of elimination is dependent on plasma concentrations, after long-term application an equilibrium concentration of the chemical in the blood is reached. Chemicals may also be stored in fat (polychlorinated pesticides such as DDT) or bone (e.g., lead). Stored chemicals usually do not cause toxic effects because of their low concentrations at the site of toxic action (receptor). After continuous application, the capacities of the storage tissues may become saturated, and xenobiotics may then be present in higher concentration in plasma and thus at the site of action; toxic responses result. Besides cumulation of the toxic agent, the toxic effect may also cumulate (Fig. 6).

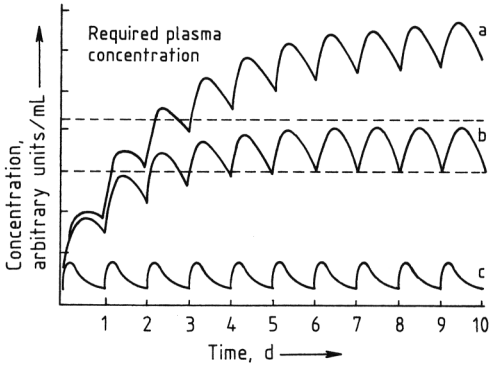


Figure 6. Accumulation of toxic chemicals based on their rate of excretion
a) The rate of excretion is equal to the rate of absorption, no accumulation occurs; b) Chemical accumulates due to a higher rate of uptake and inefficient excretion; the plasma concentrations are, however, not sufficient to exert toxic effects; c) The plasma concentrations reached after accumulation are sufficient to exert toxicity

For chemicals which irreversibly bind to macromolecules, the magnitude of toxic responses may be correlated with the total dose administered. In contrast to chemicals which act reversibly, the effect is not dependent on the frequency of dosing. Effect accumulation is often observed with carcinogens and ionizing radiation. In Figure 7 accumulation of effects is exemplified by the time- and dose-dependent induction of tumors by 4-(dimethylamino)azobenzene, a potent chemical carcinogen [5]. The TD_{50} values (50% of the treated animals carry tumors) are used to characterize the potency. Identical tumor incidences were observed after high doses and a short exposure time or after low doses and long exposure; the tumor incidence was only dependent on the total dose administered.

Reversibility of toxic responses also depends on the capacity of an organ or tissue to repair injury. For example, kidney damage by xeno-

biotics is often, after survival of the acute phase of the intoxication, without further consequence due to the high capacity of the kidney for cell proliferation and thus the capacity to repair organ damage [6]. In contrast, injury to the central nervous system is largely irreversible since the differentiated cells of the nervous system cannot divide and dead cells cannot be replaced.

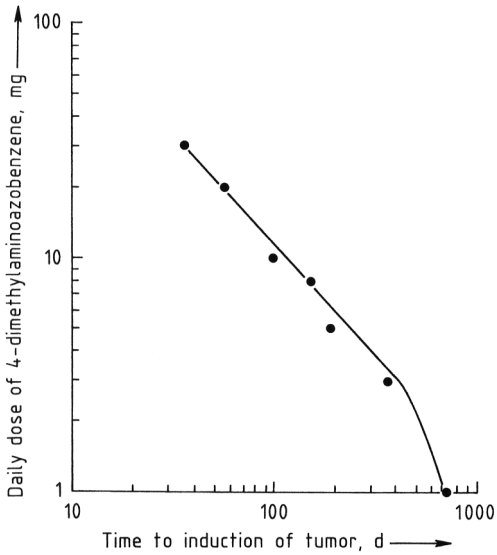


Figure 7. Time-dependent induction of tumors after different daily doses of 4-dimethylaminoazobenzene in rats [5]

1.9. Factors Influencing Dose–Response

In animals and humans, the nature, severity, and incidence of toxic responses depend on a large number of exogenous and endogenous factors [7]. Important factors are the characteristics of exposure, the species and strain of animals used for the study, and interindividual variability in humans [8]. Toxic responses are caused by a series of complex interactions of a potentially toxic chemical with an organism. The type and magnitude of the toxic response is influenced by the concentration of the chemical at the receptor and by the type of interaction with the receptor. The concentration of a chemical at the site of action is influenced by the kinetics of uptake and elimination; since these are time-dependent phenomena, toxic responses are also time-dependent. Thus, the toxic response can be separated into two phases: toxicokinetics and toxicodynamics (Fig. 8).

Toxicokinetics describe the time dependency of uptake, distribution, biotransformation, and excretion of a toxic agent (a detailed description of toxicokinetics is given in Section 2.5). Toxicodynamics describes the interaction of the toxic agent with the receptor and thus specific interactions of the agent (see below). Toxicokinetics may be heavily influenced by species, strain, and sex and the exposure characteristics [9–13]. Differences in toxic response between species, route of exposure, and others factors are often dependent on influences on toxicokinetics. Since toxicodynamics (mechanism of action) are assumed to be identical between species, this provides the basis for a rational interspecies extrapolation of toxic effects when differences in toxicokinetics are defined.

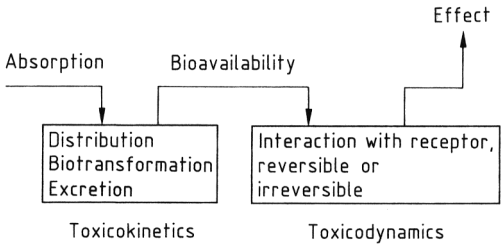


Figure 8. Toxicokinetics and toxicodynamics as factors influencing the toxic response

1.9.1. Routes of Exposure

The primary tissue or system by which a xenobiotic comes into contact with the body, and from where it may be absorbed in order to exert systemic toxicity, is the route of exposure. The frequent circumstances of environmental exposure are ingestion (peroral), inhalation, and skin contact. Also, for investigational and therapeutic purposes, intramuscular, intravenous, and subcutaneous injections may also be routes of exposure.

The major routes by which a potentially toxic chemical can enter the body are – in descending order of effectiveness for systemic delivery – injection, inhalation, absorption from the intestinal tract, and cutaneous absorption. The relationship between route and exposure, biotransformation, and potential for toxicity, may be complex and is also influenced by the magnitude and duration of dosing (Table 5).

The route of exposure has a major influence on toxicity because of the effect of route of ex-

posure on the bioavailability of the toxic agent. The maximum tissue levels achieved, the time to maximum tissue levels, and thus the duration of the effect are determined by the rate of absorption and the extent of distribution within the system.

Table 5. Toxicity of chemicals applied by different routes of exposure (data taken from [13])

Chemical	Species	Route of application	LD_{50}, mg/kg
DDT	rat	intravenous	68
	rat	oral	113
	rat	skin contact	1931
Atropine sulfate	rat	intravenous	41
	rat	oral	620
1-Chloro-2,4-dinitro-benzene	rat	oral	1070
	rat	intraperitoneal	280
	rabbit	skin contact	130
Dieldrin	rat	oral	46
	rat	intravenous	9
	rat	skin contact	10

Direct injection into veins is usually restricted to therapeutic applications, but it is important for the toxicology of intravenously injected drugs in addicts. Chemicals applied by intravenous injection are rapidly distributed to well-perfused organs in the blood and thus may result in the rapid induction of toxic effects. The rapid dilution of a chemical after intravenous injection by venous blood permits even the injection of locally acting or corrosive chemicals which are well tolerated. The likelihood of toxicity from inhaled chemicals depends on a number of factors, of which the physical state and properties of the agent, concentration, and time and frequency of exposure are important. Major influences on the absorption and disposition of xenobiotics are exerted by species peculiarities since the anatomy of the respiratory tract and the physiology of respiration show major differences between rodents and humans. The water solubility of a gaseous xenobiotic has a major influence on penetration into the respiratory tract. As water solubility decreases and lipid solubility increases, penetration into deeper regions of the lung, the bronchioli, and the alveoli becomes more effective. Water-soluble molecules such as formaldehyde,are effectively scavenged by the upper respiratory tract and may have toxic effects on the eye and throat. In contrast, gases with low water solubility such as phosgene may penetrate through the bronchii and bronchioli to the alveoli. Damage to the alveolar surface may initiate a series of events that finally results in lung edema. The degree to which inhaled gases, vapors, and particulates are absorbed, and hence their potential to produce systemic toxicity, depend on their diffusion rate through the alveolar mebrane, their solubility in blood and tissue fluids, the rate of respiration, and blood flow through the capillaries.

Uptake through the alimentary tract represents an important route of exposure for xenobiotics accumulated in the food chain, for natural constituents of human diet, and, drugs. Absorption from the gastrointestinal tract is dependent on the lipophilicity of a chemical, the molecular mass of the xenobiotic, and the presence of certain dietary constituents may influence the extent and rate of absorption. Chemicals absorbed from the gastrointestinal tract are transported to the liver via the portal vein; hepatic metabolism ("hepatic first-pass effect") may efficiently reduce the concentration of the xenobiotic available in the systemic circulation after oral uptake. Compounds undergoing bioactivation in the liver usually exhibit greater toxicity when given orally than when absorbed across the respiratory tract, due to the high proportion of material passing through the liver. In contrast, chemicals causing toxicity to extrahepatic, well-perfused organs such as the kidney often show a lower degree of toxicity to extrahepatic target organs when given orally.

Skin contact is an important route of exposure in the occupational and domestic environments. Local effects may include acute inflammation and corrosion, chronic inflammatory responses, immune-mediated reactions, and neoplasia. The percutaneous absorption of materials may also be a significant route for the absorption of systemically toxic materials. Factors influencing the percutaneous absorption of substances include skin site, integrity of skin, temperature, formulation, and physicochemical characteristics, including charge, molecular mass, and hydro- and lipophilicity.

1.9.2. Frequency of Exposure

The exposure of experimental animals may be categorized as acute, subacute, subchronic, and

chronic. Acute exposures usually last less than 24 h, and all above-mentioned routes of exposure may be applied. With chemicals of low toxicity, repeated exposures may be used. Acute inhalation exposure is usually less than 24 h; frequently 4–8 h is chosen as timescale. Repeated exposure refers to application of the chemical for less than one month (subacute), one to three months (subchronic), and more than three months (chronic). Chronic exposures to detect specific toxic effects (carcinogenicity of a chemical) may span most of the lifetime of a rodent (up to two years). Repeated exposure may be by any route; the least labor intensive route is oral, by mixing the chemical with the diet; only for specific chemicals or to simulate likely routes of exposure for humans are application in drinking water, by gastric intubation, and by inhalation applied. These are more labor-intensive and require skilled personnel and/or sophisticated techniques and thus are more expensive.

The toxic effects observed after single exposure often are different form those seen after repeated exposure. For example, inhalation of high concentration of halothane causes anesthesia in animals and humans. In contrast, long-term application of halothane in lower doses causes liver damage in sensitive species The frequency of exposure in chronic studies is important for the temporal characterisation of exposure. Chemicals with slow rates of excretion may accumulate if applied at short dosing intervals, and toxic effects may result (see Section 1.6). Also, a chemical producing severe effect when given in a single high dose may have no detectable effects when given in several smaller doses. Interspecies and strain differences in susceptibility to chemical-induced toxicity may be due to heterogeneity of populations, species specific physiology (for example of the respiratory system), basal metabolic rate, size- and species-specific toxicokinetics and routes of metabolism or excretion (Table 6). In some cases, animal tests may give an underestimate, in others an overestimate, of potential toxicity to humans [14].

1.9.3. Species-Specific Differences in Toxicokinetics

Species-specific differences in toxic response are largely due to difference in toxicokinetics and biotransformation. Distribution and elimination characteristics are quite variable between species. Both qualitative and quantitative differences in biotransformation may effect the sensitivity of a given species to a toxic response (Table 7).

Table 6. Comparative LD_{50} values for four different chemicals in different animal species and estimated LD_{50} for humans

Chemical	Species	LD_{50}, mg/kg
Paraquat	rat	134
	mouse	77
	guinea pig	41
	human	32 – 48
Ethanol	rat	12 500
	mouse	8000
	guinea pig	5500
	human	3500 – 5000
Acetaminophen	rat	3763
	mouse	777
	guinea pig	2968
	human	42 800
Aspirin	rat	1683
	mouse	1769
	guinea pig	1102
	human	3492

Table 7. Species and sex differences in the acute toxicity of 1,1-dichloroethylene after oral administration and inhalation in rats and mice (data from World Health Organization, Geneva, 1990)

Species	Dosing criteria	Estimated LD_{50}/LC_{50}
Rat, male	inhalation/4 h	7000 – 32 000 mg/L
Rat, female	inhalation/4 h	10 300 mg/L
Mouse, male	inhalation/4 h	115 mg/L
Mouse, female	inhalation/4 h	205 mg/L
Rat, male	gavage	1550 mg/kg
Rat, female	gavage	1500 mg/kg
Mouse, male	gavage	201 – 235 mg/kg
Mouse, female	gavage	171 – 221 mg/kg

For example, the elimination half-live of 2,3,7,8-tetrachlorodibenzodioxin in rats is 20 d, and in humans it is estimated to be up to seven years [15]. An example for quantitative difference in the extent of biotransformation as a factor influencing toxic response is the species differences in the biotransformation of the inhalation anesthetic halothane. Both rats and guinea pigs metabolize halothane to trifluoroacetic acid, a reaction catalyzed by a specific cytochrome P450 enzyme [16–18]. As a metabolic intermediate, trifluoroacetyl chloride is formed, which may react with lysine residues in proteins and with phosphatidyl ethanolamine in phospholipids (Fig. 9).

This interaction initiates a cascade of events finally resulting in toxicity. The metabolism of halothane in guinea pigs occurs at much higher rates than in rats, so guinea pigs are sensitive to halothane-induced hepatotoxic effects and rats are resistant. Qualitative differences in biotransformation are responsible for apparent differences in the sensitivity of rats and guinea pigs to the bladder carcinogenicity of 2-acetylamidofluorene. In rats, 2-acetylamidofluorene is metabolized by *N*-oxidation by certain cytochrome P450 enzymes. The *N*-oxide is further converted to an electrophilic nitrenium ion which interacts with DNA in the bladder; this biotransformation pathway explains the formation of bladder tumors in rats after long-term exposure to 2-acetylamidofluoren. In guinea pigs, 2-acetylamidofluorene is metabolized by oxidation at the aromatic ring; since nitrenium ions cannot be formed by this pathway, guinea pigs are resistant to the bladder carcinogenicity of 2-acetylamidofluorene (Fig. 10).

With some chemicals, age may significantly affect toxicity, likely due to age related differences in toxicokinetics. The nutritional status may modify toxic response, likely by altering the concentration of cofactors needed for biotransformation and detoxication of toxic chemicals. Diet also markedly influences carcinogen-induced tumor incidence in animals [19] and may be a significant factor contributing to human cancer incidence.

The toxic response is influenced by the magnitude, number, and frequency of dosing. Thus, local or systemic toxicity produced by acute exposure may also occur by a cumulative process with repeated exposures to lower doses; also, additional toxicity may be seen in repeated-exposure situations. The relationships for cumulative toxicity by repetitive exposure compared with acute exposure toxicity may be complex, and the potential for cumulative toxicity from acute doses may not be quantitatively predictable. For repeated-exposure toxicity, the precise profiling of doses may significantly influence toxicity.

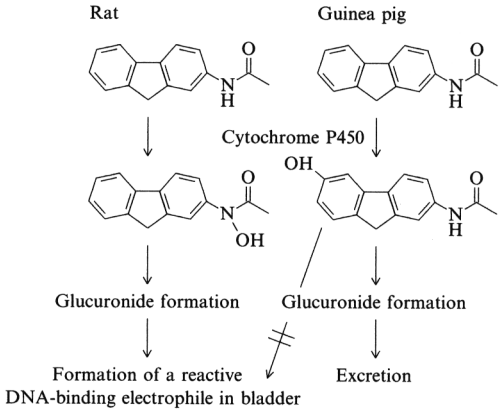


Figure 10. Biotransformation pathways of 2-acetylamidofluorene in rats and guinea pigs

1.9.4. Miscellaneous Factors Influencing the Magnitude of Toxic Responses

A variety of other factors may affect the nature and exhibition of toxicity, depending on the conditions of the study, for example, housing conditions, handling, volume of dosing, vehicle, etc. Variability in test conditions and procedures may result in significant interlaboratory variability in results of otherwise standard procedures. For chemicals given orally or applied to the skin,

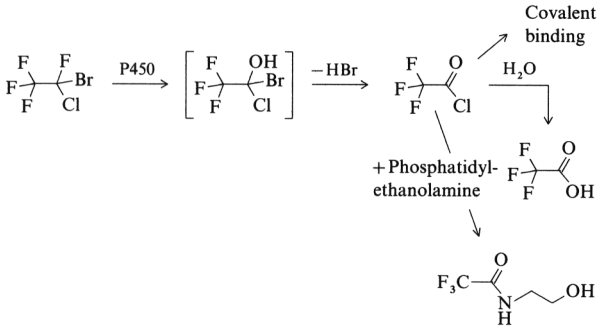


Figure 9. Halothane metabolism by cytochrome P450 in rats, guinea pigs, and humans

toxicity may be modified by the presence of materials in formulations which facilitate or retard the absorption of the chemicals. With respiratory exposure to aerosols, particle size significantly determines the depth of penetration and deposition in the respiratory tract and thus the site and extent of the toxic effects.

1.10. Exposure to Mixtures

In experimental animals most data on the toxic effects of chemicals are collected after exposure to a single chemical; in contrast, human exposure normally occurs to mixtures of chemicals at low doses. Moreover, prior, coincidential, and sucessive exposure of humans to chemicals is likely. Interactions between the toxic effects of different chemicals are difficult to predict, effects of exposure to different chemicals may be independent, additive, potentiating (ethanol and carbon tetrachloride), antagonistic (interference with action of other chemical, e.g., as seen with antidotes administered in case of intoxications), and synergistic. Ethanol exerts a potentiating effect on the hepatotoxicity of carbon tetrachloride. In rats pretreated with ethanol, the hepatotoxic effects of carbon tetrachloride are much more pronounced than in control animals. This potentiation is due to an increased capacity for bioactivation (see Section 2.4) of carbon tetrachloride in pretreated rats due to increased concentrations of a cytochrome P450 enzyme in the liver [20]. Thus, an important considerations for the assessement of potential toxic effects of mixtures of chemicals are toxicokinetics and toxicodynamic interactions. Toxicokinetic interactions of chemicals may influence absorption, distribution, and biotransformation, both to active and inactive metabolites. Mixtures of solvents often show a competitive inhibition of biotransformation. Usually, one of the components has high affinity for a specific enzyme involved in its biotransformation, whereas another component has only a low affinity for that particular enzyme. Thus, preferential biotransformation of the component with the high affinity occurs. Different outcomes of enzyme inhibition are possible: if the toxic effects of the component whose metabolism is inhibited is dependent on bioactivation, lower rates of bioactivation will result in decreased toxicity; if the toxic effects are independent of biotransformation, the extent of toxicity will increase due to slower rate of excretion. Toxic effects of mixtures may also not be due to a major component, but to trace impurities with high toxicity. For example, many long-term effects seen in animal studies on the toxicity of chlorophenols are believed to be due to 2,3,7,8-Tetrachlorodibenzodioxin, which was present as a minor impurity in the samples of chlorophenols used for these studies.

2. Absorption, Distribution, Biotransformation and Elimination of Xenobiotics

2.1. Disposition of Xenobiotics

The induction of systemic toxicity usually results from a complex interaction between absorbed parent chemical and biotransformation products formed in tissues; the distribution of both parent chemical and biotransformation products in body fluids and tissues; their binding and storage characteristics; and their excretion.

The biological effects initiated by a xenobiotic are not related simply to its inherent toxic properties; the initiation, intensity, and duration of response are a function of numerous factors intrinsic to the biological system and the administered dose. Each factor influences the ultimate interaction of the xenobiotic and the active site (Section 1.9). Only when the toxic chemical has reached the specific site and interacted with it can the inherent toxicity be realized. The route a xenobiotic follows from the point of administration or absorption to the site of action usually involves many steps and is termed toxicokinetics. Toxicokinetics influence the concentration of the xenobiotic or its active metabolite at the receptor. In the dose–response concept outlined in Section 1.9 and 1.7, it is generally assumed that the toxic response is proportional to the concentration of the xenobiotic at the receptor. However, the same dose of a chemical administered by different routes may cause different toxic effects. Moreover, the same dose of two different chemicals may result in vastly different concentrations of the chemical or its biotransformation products in a particular target organ. This differential pattern is due to differences in the disposition of a xenobiotic (Fig. 11).

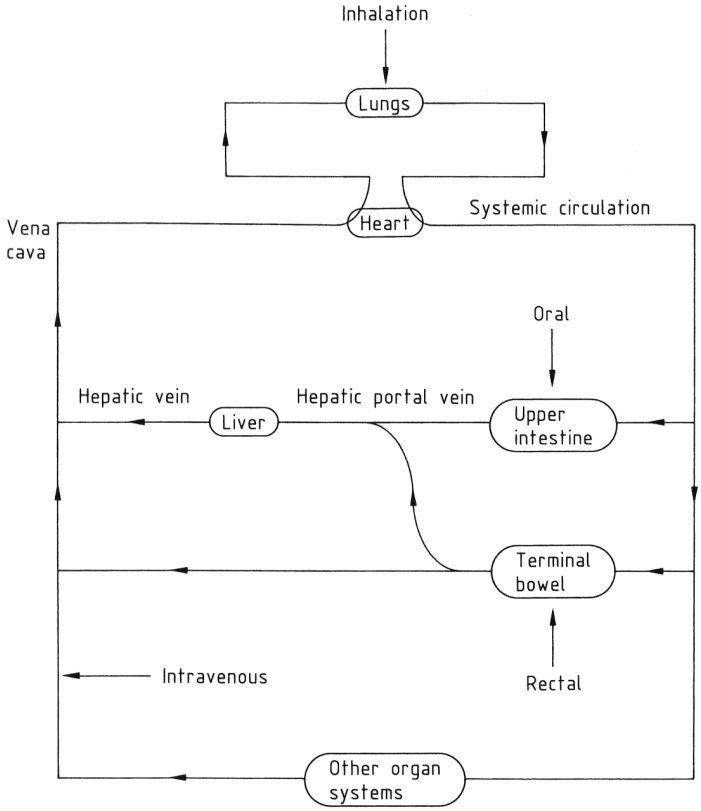


Figure 11. Possible fate of a xenobiotic in the organisms

The disposition of a xenobiotic consists of absorption, distribution, biotransformation, and excretion, which are all interrelated. The complicated interactions between the different processes of distribution are very important determinants of the concentration of a chemical at the receptor and thus of the magnitude of toxic response. They may also be major determinants for organ-specific toxicity.

For example, in the case of absorption of a xenobiotic through the gastrointestinal tract, the chemical proceeds from the intestinal lumen into the epithelial cells. Following intracellular transport, it passes through the basal membrane and lamina propria and enters the blood or lymph capillaries for transport to the site of action or storage. At that site, the xenobiotic is released from the capillaries, into an interstitial area, and finally through various membranes to its site of action, which may be a specific receptor, an enzyme, a membrane, or many other possible sites.

2.2. Absorption

The skin, the lungs, and the cells lining the alimentary tract are major barriers for chemicals present in the environment. Except for caustic chemicals, which act at the site of first contact with the organism, xenobiotics must cross these barriers to exert toxic effects on one or several target organs. The process whereby a xenobiotic moves through these barriers and enters the circulation is termed absorption.

2.2.1. Membranes

Because xenobiotics must often pass through membranes on their way to the receptor, it is important to understand membrane characteristics and the factors that permit transfer of foreign compounds. Membranes are initially encountered whether a xenobiotic is absorbed

by the dermal, oral, or vapor route. These membranes may be associated with several layers of cells or a single cell. The absorption of a substance from the site of exposure may result from passive diffusion, facilitated diffusion, active transport, or the formation of transport vesicles (pinocytosis and phagocytosis). The process of absorption may be facilitated or retarded by a variety of factors; for example, elevated temperature increases percutaneous absorption by cutaneous vasodilation, and surface-active materials facilitate penetration. Each area of entry for xenobiotics into the organism may have specific peculiarities, but a unifying concept of biology is the basic similarity of all membranes in tissues, cells, and organelles.

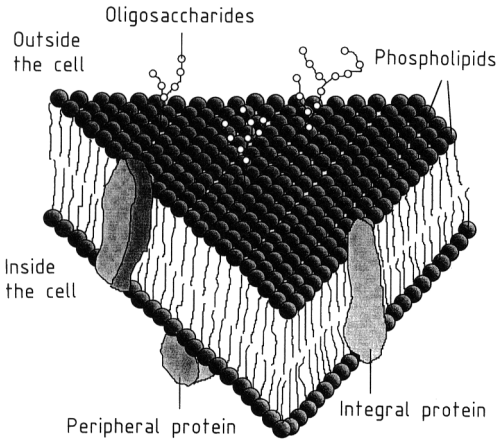


Figure 12. Simplified model of the structure of a biological membrane

All membranes are lipid bilayers with polar head groups (phosphatidylethanolamine, phosphatidylcholine). The polar groups predominate at the outer and inner surfaces of the membrane; the inner space of the membrane consists of perpendicularly arranged fatty acids [21]. The fatty acids do not have a rigid structure and are fluid under physiological conditions; the fluid character of the membrane is largely dominated by the fatty acid composition. The width of a biological membrane is approximately 7–9 nm. Figure 12 illustrates the concept of a biological membrane (fluid-mosaic model).

Proteins are intimately associated with the membrane and may be located on the surface or inside the membrane structure, or extend completely through the membrane. These proteins may also form aqueous pores. Hydrophobic forces are responsible for maintaining the structural integrity of both proteins and lipids within the membrane structure. The ratio of lipid to protein in different membranes may vary from 5:1 (e.g,. myelin) to 1:5 (e.g., the inner membrane of mitochondria). Usually, pore diameters in membranes are small and permit only the passage of low molecular mass chemicals. However, some specialized membranes such as those found in the glomeruli of the kidney, which can have pore sizes of up to 4 nm, also permit the passage of compounds with molecular mass greater than 10 000.

The amphipathic nature of the membrane creates a barrier for ionized, highly polar compounds; however, changes in lipid composition, alterations in the shape and size of proteins, and physical features of bonding may cause changes in the permeability of membranes [22].

2.2.2. Penetration of Membranes by Chemicals

A chemical can pass through a membrane by two general processes: passive diffusion and active transport. Passive diffusion is described by Fick's law and requires no energy. Active transport processes involve the consumption of cellular energy to translocate the chemical across the membrane. Active transport may also act against a concentration gradient and result in the accumulation of a xenobiotic in a specific organ, cell type or organelle.

Diffusion of Chemicals through Membranes. Many toxic chemicals pass membranes by simple diffusion. Their rates of diffusion depend on their lipid solubility and are often correlated with the partition coefficient (solubility in organic solvents/solubility in water). Lipophilic chemicals may diffuse directly through the lipid domain of the membrane. However, a certain degree of water solubility seems to be required for passage since many poorly lipid soluble chemicals have been shown to penetrate easily. Once initial penetration has occurred, the molecule must necessarily traverse a more polar region to dissociate from the membrane. Compounds with extremely high partition coefficients thus tend to remain in membranes and to accumulate there rather than pass through them. Polar com-

pounds that are insoluble in the nonpolar, fatty-acid-containing inner space of the membrane often cannot penetrate membranes, although some low molecular mass polar chemicals may slowly penetrate through the aqueous pores of the membranes.

The rates of movement of nonpolar xenobiotics through membranes can be predicted based on the assumptions from Fick's law of diffusion. Polar compounds and electrolytes of low molecular mass are believed to behave similarily. A first-order equation appears to be applicable to the majority of xenobiotics. The rate of diffusion of a xenobiotic is related to its concentration gradient across the membrane ($C_1 - C_2$), the surface area available for transfer A, the diameter of the membrane d, and the diffusion constant k. The latter is related to the size and structure of the molecule, the spatial configuration of the molecule, and the degree of ionization and lipid solubility of the xenobiotic.

$$\text{Rate of diffusion} = k\,\frac{A\,(C_1 - C_2)}{d}$$

As the xenobiotic is rapidly removed after absorption, C_2 can usually be ignored. and a log/linear plot of the amount of unpenetrated chemicals present over time should be linear. When relatively comparable methods have been used, calculation of the half-time of penetration $t_{1/2}$, is useful. The rate constant of penetration k is derived from

$$k = \frac{0.693}{t_{1/2}}$$

When the half-time of penetration after oral and dermal administration of several environmental contaminants were compared, rates were found to vary considerably. Clearly, rates of penetration by different routes in mammals show little or no correlation.

Ionization becomes particularly important when xenobiotics are introduced into the gastrointestinal tract, where a variety of pH conditions are manifest (see Section 2.2.4.2). Although many drugs are acids and bases and thus potentially ionizable form, most xenobiotics are neither acids nor bases and thus are unaffected by pH. The amount of a xenobiotic in the ionized or unionized form depends upon the pK_a of the xenobiotic and the pH of the medium. When the pH of a solution is equal to the pK_a of the dissolved compound, 50% of the acid or base exists in the ionized and 50% in the unionized form. The degree of ionization at a specific pH is given by the Henderson–Hasselbalch equation:

$$pK_a - \text{pH} = \log\frac{[\text{nonionized}]}{[\text{ionized}]}$$

$$pK_a - \text{pH} = \log\frac{[\text{ionized}]}{[\text{nonionized}]}$$

Since the unionized, lipid-soluble form of a weak acid or base may penetrate membranes, weak organic acids diffuse most readily in an acidic environment, and organic bases in a basic environment. There is some degree of penetration even when xenobiotics are not in the most lipid-soluble form, and a small amount of absorption can produce serious effects if a compound is very toxic.

2.2.3. Mechanisms of Transport of Xenobiotics through Membranes

Filtration. Passage of a solution across a porous membrane results in the retention of solutes larger than the pores. This process is termed filtration. For example, filtration of solutes occurs in the kidney glomeruli, which have large pores and retain molecules with molecular masses greater than 10 000. Elsewhere in the body, filtration by pores may only result in the passage of relatively small molecules (molecular mass ca. 100), and most larger molecules are excluded. Thus, uptake of xenobiotics through these pores is only a minor mechanism of penetration.

Special Transport Mechanisms. Special transport processes include active transport, facilitated transport, and endocytosis (Table 8). Often, the movement of chemicals across membranes is not due to simple diffusion or filtration. Even some very large or very polar molecules may readily pass through membranes.

Active transport systems have frequently been implicated in these phenomena. *Active transport* may be effected by systems that help

Table 8. Special transport processes involved in the passage of xenobiotics through biological membranes

Type of transport	Carrier molecule required	Examples of substrates	Energy required	Against concentration gradient
Active transport	yes	organic acids in the kidney	yes	yes
Facilitated transport	yes	glucose	yes	no
Endocytosis	no	proteins	yes	?

transport endogenous compounds across membranes. Such processes require energy and transport xenobiotics against electrochemical or concentration gradients. Active transport systems are saturable processes and exhibit a maximum rate of transport; they are usually specific for certain structural features of chemicals. A carrier molecule (likely a protein) associates with the chemical outside the cell, translocates it across the membrane for ultimate release inside the cell. This is particularly important for compounds that lack sufficient lipid solubility to move rapidly through the membrane by simple diffusion. Active transport plays a major role in the excretion of xenobiotics from the body, and major excretory organs such as the liver or the kidney have several transport systems which may accept organic acids, organic bases, or even metal ions as substrates.

In contrast to other special transport processes, some carrier-mediated processes do not require energy and are unable to move chemicals against a concentration gradient. These processes are termed *facilitated transport*. Facilitated transport is particulary beneficial for compounds which lack sufficient lipid solubility for rapid diffusion through the membrane. Facilitated transport is more rapid than simple diffusion up to the point at which concentrations on both sides of the membranes are equal. For example, the transport of glucose through a variety of membranes occurs by facilitated transport. The mechanisms by which facilitated transport occurs are not well understood.

Pinocytosis (liquids) and *phagocytosis* (solids) are specialized processes in which the cell membrane invaginates or flows around a xenobiotic, usually present in particulate form, and thus enables transfer across a membrane. Although of importance once the xenobiotic has gained entry into the organism, this mechanism does not appear to be of importance in the initial absorption of a xenobiotic.

2.2.4. Absorption

Absorption is the process whereby xenobiotics cross body membranes and are translocated to the blood stream. The primary sites of absorption of environmental contaminants are the gastrointestinal tract (gastrointestinal absorption), the skin (dermal absorption), and the lung (respiratory absorption). Absorption of chemicals may also occur from other sites such as muscle, the subcutis, or the peritoneum after administration by special routes. In clinical medicine, many drugs are injected directly into the bloodstream to circumvent the problems of absorption posed by the peculiarities of the different routes.

2.2.4.1. Dermal Absorption

Human skin can come into contact with many potentially toxic chemicals. Skin is relatively impermeable to aqueous solutions and most xenobiotics present as ions. Therefore, it is a relatively good barrier separating the human body from the environment. However, skin is permeable in varying degrees to a large number of xenobiotics, and some chemicals may be absorbed through the skin in sufficient amounts to cause a toxic response [23]. A striking example of the significance of absorption through the skin is the large number of agricultural workers who have experienced acute poisoning from exposure to parathion (dermal $LD_{50} \approx 20$ mg/kg) during application or from exposure to vegetation previously treated with this pesticide.

The human skin is a complex, multilayered tissue with approximately 18 000 cm^2 of surface in an average human male. Chemicals to be absorbed must pass through several cell layers before entering the small blood and lymph capillaries in the dermis. Transport in blood and lymph then distributes absorbed chemicals in the

body. The human skin consists of three distinct layers (Fig. 13) and a number of associated appendages (sweat and sebaceous glands, hair follicles).

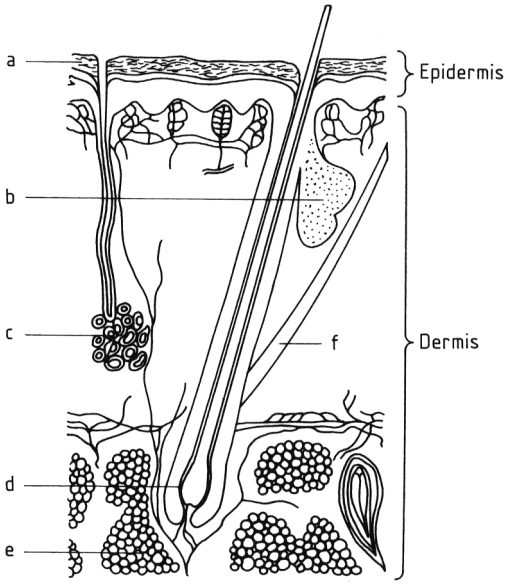


Figure 13. Cross section of human skin
a) Stratum corneum; b) Sebaceous gland; c) Sweat gland; d) Hair follicle; e) Fat; f) Muscle

The *epidermis* is a multilayered tissue varying in thickness from 0.15 (eyelids) to 0.8 mm (palms). This tissue appears to be the greatest deterrent to the absorption of xenobiotics. The epithelial tissues of the skin develop and grow divergently from other tissues. Proliferative layers of the basal cells (stratum germinativum) differentiate and gradually replace cells above them as surface cells deteriorate and are sloughed from the epidermis. Cells in this layer produce fibrous, insoluble keratin that fills the cells, and a sulfur-rich amorphous protein that comprises the cell matrix and thickened cell membrane. This cell layer, the stratum corneum, provides the primary barrier to the penetration of foreign compounds. It consists of several layers of flattened, stratified, highly keratinized cells. These cells are approximately 25–40 μm wide and have lost their nuclei. Although highly water retarding, the dead, keratinized cells of the stratum corneum are highly water absorbent (hydrophilic), a property that keeps the skin supple and soft. A natural oil covering the skin, the sebum, appears to maintain the water-holding capacity of the epidermis but has no appreciable role in retarding the penetration of xenobiotics. The rate-determining barrier in the chemical absorption of xenobiotics is the stratum corneum.

The *dermis* and *subcutaneous tissue* offer little resistance to penetration, and once a substance has penetrated the epidermis these tissues are rapidly traversed. The dermis is a highly vascular area that provides ready access to blood and lymph for distribution once the epithelial barrier has been passed. The blood supply in the dermis is subjected to complex, interacting neural and humoral influences whose temperature-regulating function can have an effect on distribution by altering blood supply to this area. Therefore, the extent of absorption of a chemical through the skin may be influenced by temperature, and relative humidity [24].

The *skin appendages* are found in the dermis and extend through the epidermis. The primary appendages are the sweat glands (epicrine and apocrine), hair, and sebaceous glands. These structures extend to the outer surface and therefore may play a role in the penetration of xenobiotics; however, since they represent only 0.1 to 1% of the total surface of the skin, their contribution to overall dermal absorption is usually minor.

Percutaneous absorption can occur by several routes, but the majority of unionized, lipid-soluble xenobiotics appear to move by passive diffusion directly through the cells of the stratum corneum. Important arguments for the importance of transepidermal absorption are that epidermal damage or removal of the stratum corneum increases permeability, the epidermal penetration rate equals whole-skin penetration, epidermal penetration is markedly slower than dermal, and the epidermal surface area is 100–1000 times the surface area of the skin appendages. Very small and/or polar molecules appear to have more favorable penetration through appendages or other diffusion shunts, but only a small fraction of toxic xenobiotics are chemicals of this type. Polar substances, in addition to movement through shunts, may diffuse through the outer surface of the protein filaments of the hydrated stratum corneum, while nonpolar molecules dissolve in and diffuse through the nonaqueous lipid matrix between the protein filaments.

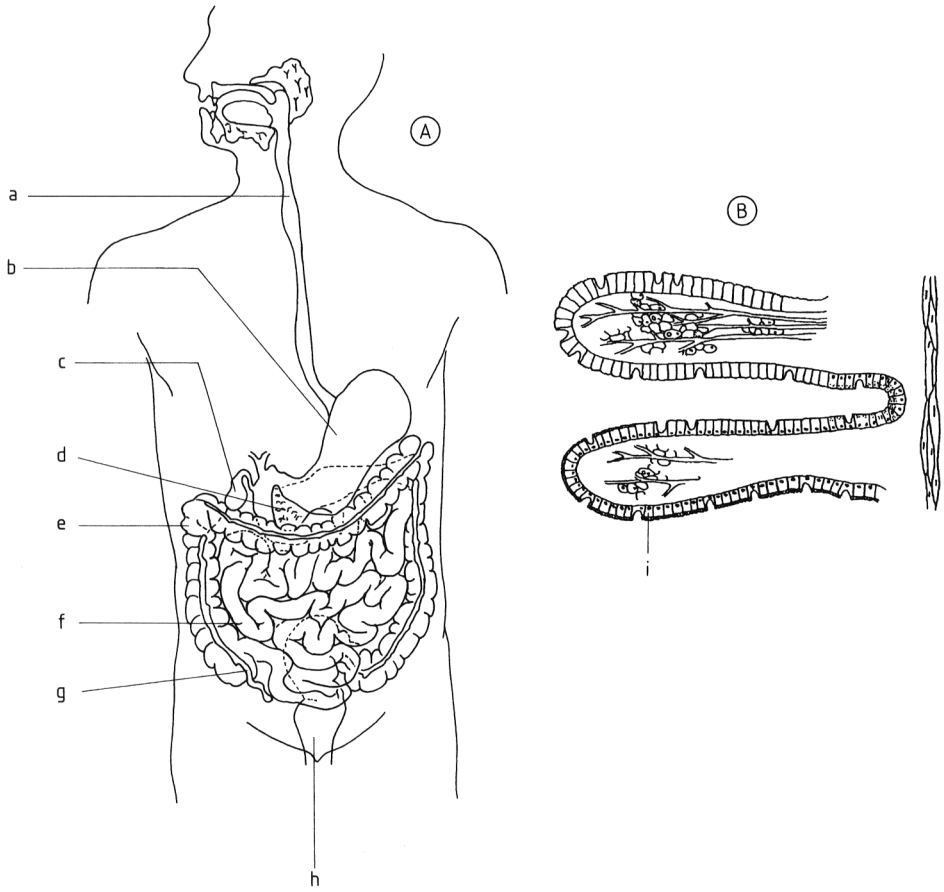


Figure 14. A) Intestinal tract in humans; B) Anatomy of the intestinal wall, the major site of absorption of xenobiotics
The lining of the small intestine is highly folded and has a special surface structure (brush-border membrane) to give a large surface available for the efficient uptake of nutrients.
a) Esophagus (4 – 7.2); b) Stomach (1.0 – 3.0); c) Duodenum (4.8 – 8.2); d) Pancreas; e) Colon (7.9 – 8.0); f) Jejunum (7.6); g) Ileum (7.6); h) Rectum (7.8); i) Brush-border membrane
Numbers in brackets represent pH in different parts of the intestinal tract.

Human stratum corneum displays significant differences in structure from one region of the body to the other, which affect the rate of absorption. Penetration at certain body regions thus varies according to the polarity and size of the molecule, but it is generally accepted that for most unionized xenobiotics the rate of penetration is in the following order: scrotal > forehead > axilla = scalp > back = abdomen > palm and plantar. The palm and plantar regions are highly diffuse, but their much greater thickness (100–400 times that of other regions) introduces an overall lag time in diffusion.

The condition of the skin greatly influences the absorption of xenobiotics. Damage to or removal of the stratum corneum cause a dramatic increase in the permeability of the epidermis for xenobiotics. Caustic and corrosive chemicals such as acids or alkali or burns will greatly enhance dermal absorption and thus influence the toxicity of a xenobiotic applied to the skin. Soaps and detergents are among the damaging substances routinely applied to skin. Whereas organic solvents must be applied in high concentrations to damage skin, 1% aqueous solutions of detergents increase the rate of penetration of solutes through human epidermis dramatically. Alteration of the stratum corneum by organic solvents may also be the cause of increased penetration.

Organic solvents can be divided into damaging and nondamaging categories. Damaging solvents include methanol, acetone, diethyl ether, hexane, and some solvent mixtures. These solvents and mixtures can extract lipids and proteolipids from tissues and are thus expected to alter permeability. Although the mechanical strength of the stratum corneum is unaltered, delipidization produces a more porous, nonselective surface. Solvents such as higher alcohols, esters, and olive oil do not appear to damage skin appreciably. On the contrary, the penetration rate of solutes dissolved in them is often reduced. Surprisingly, lipid-soluble xenobiotics may be markedly resistant to washing, even a short time after application. For example, 15 min after application, a substantial portion of parathion cannot be removed from contaminated skin by soap and water.

When comparisons across species are made, human skin appears to be more impermeable, or at least as impermeable, as the skin of the cat, dog, rat, mouse, or guinea pig. The skin of pigs and guinea pigs in particular serves as a useful approximation to human skin, but only after a comparison has been made for each specific chemical.

Temperature, surface area of applied dose, simultaneous application of another xenobiotic, relative humidity, occlusion, age, and hyperthermia are among a number of chemical, physical, and physiological factors that may alter skin penetration.

2.2.4.2. Gastrointestinal Absorption

The oral route of entry into the body is specially important for accidental or purposeful (suicide) ingestion of poisonous materials. Food additives, food toxins, environmental xenobiotics accumulated in the food chain, and airborne particles excluded from passage to to alveoli are also introduced into the digestive system. The penetration of orally administered xenobiotics is primarily confined to the stomach and intestine [25].

The gastrointestinal tract may be viewed as a tube traversing the body. It consists of the mouth, esophagus, stomach, small and large intestine, colon, and rectum (Fig. 14). The digestive tract is lined by a single layer of columnar cells, usually protected by mucus, which do not present a barrier to penetration. The circulatory system is closely associated with the intestinal tract (30–50 μm from membrane to vasculature), and once xenobiotics have crossed the epithelium of the intestinal tract, entry into capillaries is rapid. Venous blood flow from the stomach and intestine rapidly removes absorbed xenobiotics and introduces them into the hepatic portal vein, which transports them to the liver.

Absorption of chemicals may take place along the entire gastrointestinal tract, but most xenobiotics are absorbed in the stomach and the small intestine. A major factor favoring absorption in the intestine is the presence of microvilli that increase the surface area to an estimated 100 m^2 in the small intestine (see Fig. 14) Because the intestinal area thus offers maximal opportunity for absorption, it is generally accepted that absorption of xenobiotics is greatest in this area of the gastrointestinal tract. Although the gastrointestinal tract has some special transport processes for the absorption of nutrients and electrolytes, most xenobiotics seem to enter the body from the gastrointestinal tract by simple diffusion. Exeptions are some heavy metals such as thallium and lead, which mimic the essential metals iron and calcium, respectively. They are thus absorbed by active transport systems developed for the uptake of these nutrients.

The gastrointestinal tract has areas of highly variable pH, which can markedly change the permeability characteristics of ionic compounds. For example, passive diffusion is greatly limited except for unionized, lipid-soluble chemicals. Although variable according to secretory activity, the pH of the stomach is ca. 1–3 and that of the intestine ca. 7. The measured pH of the intestinal contents may not be the same as the pH of the epithelium at the site of absorption, and this explains the entrance of compounds whose pKa would suggest less favored absorption. The variations in pH in the different sections of the intestinal tract may influence the absorption of acids and bases. Since most xenobiotics are absorbed by diffusion, only the unionized, membrane-permeable form may be absorbed. Weak organic acids are mainly present in the unionized, lipid-soluble form in the stomach, and predominantly in the ionized form in the intestine. Therefore, organic acids are expected to be more readily absorbed from the stomach than from

the intestine. In contrast, weak organic bases are ionized in the stomach but present in the lipid-soluble form (unionized) in the intestine. Absorption of such compounds should therefore predominantly occur in the intestine rather than in the stomach.

However, other factors determining the rate of membrane penetration such as surface area available for diffusion, blood flow (influencing concentration gradients), and the law of mass action also influence the site of absorption of acids or bases from the gastrointestinal tract. For example, although only 1% of benzoic acid is present in the lipid-soluble, unionized form in the small intestine, the large surface area and the rapid removal of absorbed benzoic acid with the blood result in its efficient absorption from the small intestine.

Other factors contribute to gastrointestinal absorption Clearly a xenobiotic must be dissolved before absorption can take place. Particle size, organic solvents, emulsifiers, and rate of dissolution thus also effect absorption. In addition, the presence of microorganisms and hydrolysis-promoting pH offer opportunities for the biotransformation of many xenobiotics. Other factors affecting gastrointestinal absorption include binding to gut contents, intestinal motility, rate of emptying, temperature of food, effects of dietary constituents, health status of the individual, and gastrointestinal secretion.

2.2.4.3. Absorption of Xenobiotics by the Respiratory System

The respiratory system is an organ in direct contact with environmental air as an unavoidable part of living. A number of xenobiotics exist in gaseous (carbon monoxide, nitric oxides), vapor (benzene, carbon tetrachloride), and aerosol (lead from automobile exhaust, silica, asbestos) forms and are potential candidates for entry via the respiratory system. Indeed, the most important cause of death from acute intoxication (carbon monoxide) and the most frequent occupational disease (silicosis) are caused by the absorption or deposition of airborne xenobiotics in the lung.

The respiratory tract consists of three major regions: the nasopharyngeal, the tracheobronchial, and the pulmonary (Fig. 15). The nasopharynx begins in the mouth and extents down to the level of the larynx. The trachea, bronchii, and bronchioli serve as conducting airways between the nasopharynx and the alveoli, the site of gas exchange between the inhaled air and the blood. The human respiratory system is a complex organ containing over 40 different cell types. These cell types contribute to the pulmonary architecture and function over various zones of the lung, although to some extent, individual cell types can be found in several zones. The tracheobronchial system comprises airways lined with bronchial epithelium with associated submucosal glands and several different tissues with specific function and the lung vasculature.

The absorption of xenobiotics by the respiratory route is favored by the short path of diffusion, large surface area (50–100 m^2), and large concentration gradients. At the alveoli (site of gas exchange), the membranes are very thin (1–2 μm) and are intimately associated with the vascular system. This enables rapid exchange of gases (ca. 5 ms for CO_2 and ca. 200 ms for O_2). A thin film of fluid lining the alveolar walls aids in the initial absorption of xenobiotics from the alveolar air. Simple diffusion accounts for the somewhat complex series of events in the lung regarding gas absorption. The sequences of respiration, which involve several interrelated air volumes, define both the capacity of the lung and factors important to particle deposition and retention. Among the elements important in total lung capacity is the residual volume, that is, the amount of air retained by the lung despite maximal expiratory effort. Largely due to slow release from this volume, gaseous xenobiotics in the expired air are not cleared immediately, and many expirations may be necessary to rid the air in the lung of residual xenobiotic. The rate of entry of vapor-phase xenobiotics is controlled by the alveolar ventilation rate, and a xenobiotic present in alveolar air may come into contact with the alveoli in an interrupted fashion about 20 times per minute. The diffusion coefficient of the gas in the fluids of pulmonary membranes is another important consideration, but doses are more appropriately discussed in terms of the partial pressure of the xenobiotic in the inspired air. On inhalation of a constant tension of a gaseous xenobiotic, arterial plasma tension of the gas approaches the tension of gas in the expired air. The rate of entry is then determined by blood solubil-

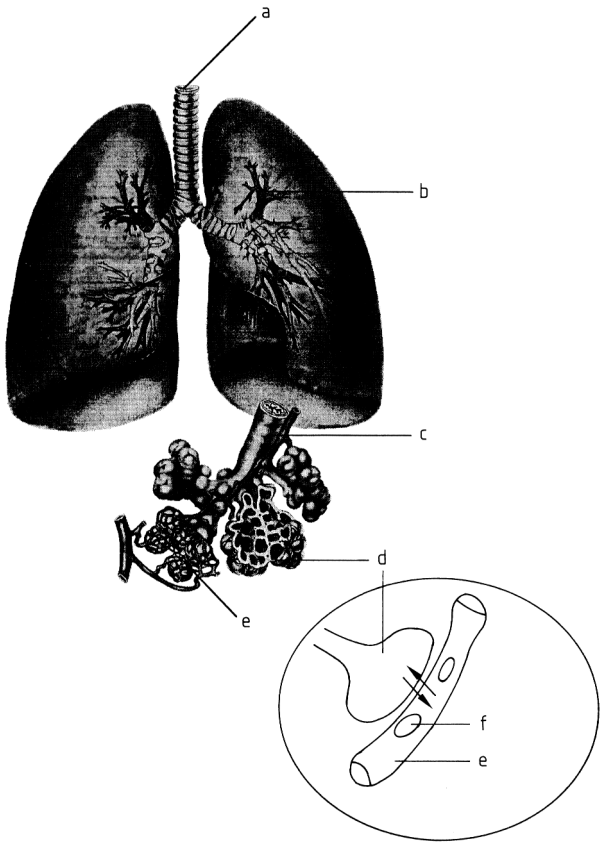


Figure 15. Anatomy of the human respiratory system
a) Trachea; b) Bronchii; c) Bronchioli; d) Alveoli; e) Capillary; f) Erythrocyte

ity of the xenobiotic and blood flow. For a high blood/gas partition coefficient, a larger amount must be dissolved in the blood to raise the partial pressure. Chemicals with a high blood/gas partition coefficient require a longer period to approach the same tension in the blood as in inspired air than less soluble gases.

Aerosols and Particulates. The entry of aerosols and particulates is affected by a number of factors. A coal miner inhales ca. 6000 g of coal dust particles during his occupational lifetime, and only ca. 100 g are found postmortem; therefore, effective protective mechanisms are operative. The parameters of air velocity and directional changes in air flow favor impaction of particles in the upper respiratory systems. Particle characteristics such as size, chemistry of the inhaled material, sedimentation and electrical charge are important to retention, absorption, or expulsion of airborne particles. In addition to the other aforementioned lung characteristics, a mucous blanket propelled by ciliary action clears the respiratory tract of particles by directing them to the gastrointestinal system (via the glottis) or to the mouth for expectoration. This system is responsible for 80% of lung particulate clearance. The deposition of various particle sizes in different respiratory regions is summarized in Figure 16, which shows that particle size is important for disposition and particles larger than 2 μm do not reach the alveoli [26].

The direct penetration of airborne xenobiotics at alveolar surfaces or in the upper respiratory tract is not the only action of toxicological importance. Both vapors and particulates can accumulate in upper respiratory passages to produce irritant effects. Irritant gases may be deposited in the respiratory tract depending on their water solubility and may cause localized

damage characterized by edema, swelling, mucus production, and increased d vascular permeability. If major airways are obstructed by these processes or important anatomical structures of the respiratory tract like the alveoli are damaged, life-threatening or deadly intoxications may be caused by the inhalation of irritant gases.

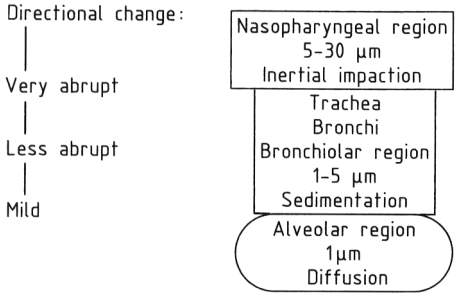


Figure 16. Effect of size on the disposition and sedimentation of particulates in the respiratory tract
The site of particle sedimentation is determined largely by particle size; only very fine particles are deposited in the alveoli; larger particles do not reach the lung but are deposited in the nasopharynx.

Despite the effectiveness of ciliary movement and phagocytosis, the cumulative effects of silica, asbestos, or coal dust ultimately cause chronic fibrosis even though direct absorption is of minor importance. Thus, phagocytosis prevents acute damage but may contribute to chronic toxicity. There is little evidence for active transport in the respiratory system, although pinocytosis may be of importance for penetration. The lung is an area of extensive metabolic activity; enzymes present in the lung may catalyze both activation and detoxication of xenobiotics (see Section 2.4).

2.3. Distribution of Xenobiotics by Body Fluids

After entering the blood by absorption or by intravenous administration, xenobiotics are available for distribution throughout the body. The initial rate of distribution to organs and tissues is determined by the blood flow to that organ and the rate of diffusion of the chemical into the specific organ or tissue. Uptake of xenobiotics into organs or tissues may occur by either passive diffusion or by special transport processes. Within tissues binding, storage, and/or biotransformation may occur. After reaching equilibrium, the distribution of a chemical among organs and tissues is largely determined by affinity; blood flow determines distribution only during the initial phase shortly after uptake.

Body fluids are distributed between three distinct compartments: vascular water, interstitial water and intracellular water. Plasma water and interstitial water are extracellular water. Plasma water plays an important role in the distribution of xenobiotics. Human plasma accounts for about 4% of the total body weight and 53% of the total volume of blood. By comparison, the interstitial tissue fluids account for 13% of body weight, and intracellular fluids account for 41%. The concentration of a xenobiotic in blood following exposure will depend largely on its apparent volume of distribution. If the xenobiotic is distributed only in the plasma, a high concentration will be achieved within the vascular tissue. In contrast, the concentration will be markedly lower if the same quantity of xenobiotic were distributed in a larger pool including the interstitial water and/or intracellular water.

Among the factors that affect distribution, apart from binding to blood macromolecules, are the route of administration, rate of biotransformation, polarity of the parent xenobiotic or biotransformation products, and rate of excretion by the liver or kidneys. Gastrointestinal absorption and intraperitoneal administration provide for immediate passage of a compound to the liver, whereas dermal or respiratory routes involve at least one passage through the systemic circulation prior to reaching the liver. The metabolism of most xenobiotics results in products that are more polar and thus more readily excreted than the parent molecules. Therefore, the rate of metabolism is a critical determinant in the distribution of a compound, since compounds that are readily metabolized are usually readily excreted, and thus are proportionally less prone to accumulate in certain tissues. The same principle applies to polarity, since very polar xenobiotics will be readily excreted. Chemicals may circulate either free or bound to plasma protein or blood cells; the degree of binding and factors influencing the equilibrium with the free form may influence availability for biotransformation, storage, and/or excretion [27].

Patterns of xenobiotic distribution reflect certain physiological properties of the organism

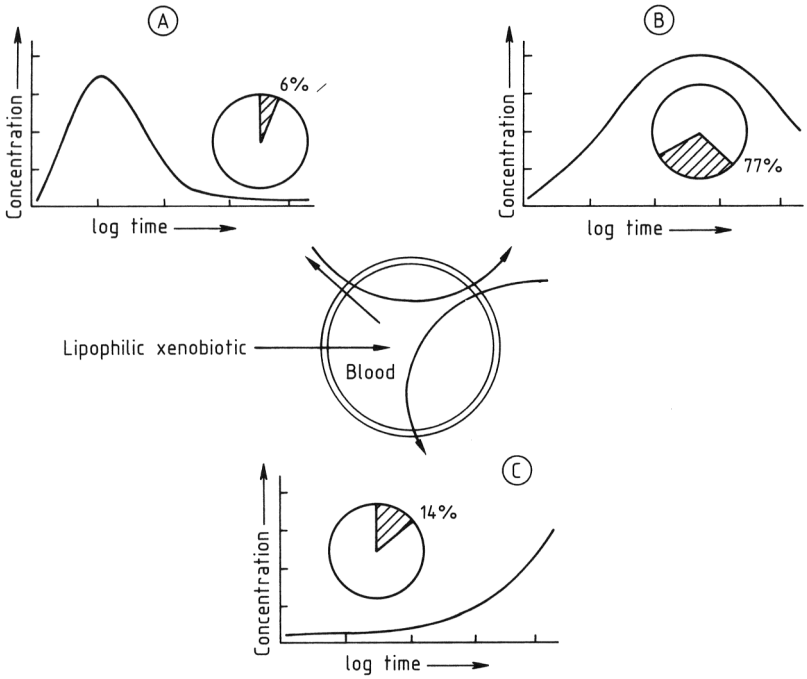


Figure 17. Uptake and redistribution with blood of lipophilic xenobiotics
Lipophilic xenobiotics in the blood are first distributed to well-perfused organs (A); after some time, they are redistributed to organs with lower blood flow representing a larger fraction of the body weight (B, C)

and the physicochemical properties of the xenobiotics. An initial phase of distribution may be distinguished that reflects cardiac output and blood flow to organs. Heart, liver, kidney, brain, and other well-perfused organ- receive most of a lipophilic xenobiotic within the first few minutes after absorption. Delivery to the smooth muscles, most viscera, and skin is slower, and the time to reach a steady-state concentration of a xenobiotic in these organs may be several hours. A second phase of xenobiotic distribution may therefore be distinguished; it is limited by blood flow to an organ or tissue and involves a far larger fraction of body mass than the first phase of distribution (Fig. 17).

Only a limited number of xenobiotics have sufficient solubility in blood to account for simple dissolution as a route of distribution; the distribution of many xenobiotics occurs in association with plasma proteins. The binding of drugs to plasma proteins is of key importance in transport. Many organic and inorganic compounds of low molecular mass appear to bind to lipoproteins, albumins, and other proteins in plasma and are transported as protein conjugates. This binding is reversible. Cellular components may also be responsible for transport of xenobiotics, but such transport is rarely a major route. The transport of xenobiotics by lymph is usually quantitatively of little importance since the intestinal blood flow is 500–700 times greater than the intestinal lymph flow.

A large number of studies on binding of drugs by plasma protein have demonstrated that binding to serum albumin is particularly important for these chemicals. Only few studies on the reversible binding of toxic xenobiotics have been performed, but available evidence suggests a significant role of lipoproteins in plasma. These plasma proteins may bind xenobiotics as well as some physiological constituents of the body. Examples for plasma proteins which may bind xenobiotics are albumin, α- and β-lipoproteins, and metal-binding proteins such as transferrin. Lipoproteins are important for the transport of lipid-soluble endogenous chemicals such as vitamins, steroid hormones, and cholesterol, but they may also bind lipophilic xenobiotics. If a

xenobiotic is bound to a protein, it is immobilized remote from the site of action. The extent of binding to plasma proteins varies considerably among xenobiotics. While some are not at all bound, for others more than 90% of administered dose may be bound to plasma proteins. These ligand–protein interactions are reversible and provide a remarkably efficient means for transport of xenobiotics to various tissues.The xenobiotic–protein interaction may be simply described according to the law of mass action as:

$$[T]_F + [\text{free sites}] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [T]_B$$

where $[T]_F$ and $[T]_B$ are the concentrations of free and bound xenobiotic molecules, respectively, and k_1 and k_2 are the rate constants for association and dissociation; k_2, which governs the rate of binding to the protein, dictates the rate of xenobiotic release at a site of action or storage. The ratio k_1/k_2 is identical with the dissociation constant K_{diss}. Among a group of binding sites on proteins, those with the smallest K_{diss} for a given xenobiotic will bind it most tightly.

In contrast to the covalent binding to proteins seen with many xenobiotics or their electrophilic metabolites (see Section 2.5.6.6), the interaction of xenobiotics with plasma proteins is most often noncovalent and reversible. Noncovalent binding is of primary importance with respect to distribution because of the opportunities to dissociate after transport. Binding of xenobiotics to plasma proteins may be due to several types of interactions which are summarized in the following.

Ionic Binding. Electrostatic attraction occurs between two oppositely charged ions. The degree of binding varies with the chemical nature of each compound and the net charge. Dissociation of ionic bonds usually occurs readily, but some transition metals exhibit high association constants (low K_{diss} values), and exchange is slow. Ionic interactions may also contribute to binding of alkaloids with ionizable nitrogen groups and other ionizable xenobiotics.

Hydrogen Bonding. Generally, only the most electronegative atoms form stable hydrogen bonds. Protein side chains containing hydroxyl, amino, carboxyl, imidazole, and carbamyl groups may form hydrogen bonds, as can the nitrogen and oxygen atoms of peptide bonds. Hydrogen bonding plays an important role in the structural configuration of proteins and nucleic acids.

Van der Waals forces are very weak interactions between the nucleus of one atom and the electrons of another atom, i.e., between dipoles and induced dipoles. The attractive forces are based on slight distortions induced in the electron clouds surrounding each nucleus as two atoms come close together. The binding force is critically dependent upon the proximity of interacting atoms and diminishes rapidly with distance. However, when these forces are summed over a large number of interacting atoms that "fit" together spatially, they can play a significant role in determining specificity of xenobiotic–protein interactions.

Hydrophobic Interactions. When two nonpolar groups come together they exclude the water between them, and this mutual repulsion of water results in a hydrophobic interaction. In the aggregate they represent the least possible disruption of interactions among polar water molecules and thus can lead to stable complexes. Some consider this a special case of van der Waals forces. The minimization of thermodynamically unfavorable contact of a polar group with water molecules provides the major stabilizing effect in hydrophobic interactions.

Consequences of the binding to plasma proteins are reduced availability of the free xenobiotic in the cells and a delayed excretion. The xenobiotic bound to plasma protein cannot cross capillary walls due to its high molecular mass. The fraction of dose bound is thus not available for delivery to the extravascular space or for filtration by the kidney. It is generally accepted that the fraction of xenobiotic that is bound may not exert toxic effects; however, many xenobiotics and endogenous compounds appear to compete for the same binding site, and thus one compound may alter the unbound fraction of another by displacement, thereby potentially increasing toxic effects. Plasma proteins that can bind endogenous chemicals and xenobiotics are listed below, together with examples of bonded xenobiotics:

α-Lipoproteins	vitamins A, K, D
	steroid hormones
	dieldrin
Albumin	salicylate
	tetracyclines
	phenols
	vitamin C

Binding of xenobiotics to plasma proteins is mostly reversible; the bound xenobiotic is in equilibrium with free xenobiotic, and thus binding usually slows excretion or delivery to cellular sites of action. Toxicological consequences of the reversible binding of a xenobiotic to plasma proteins may arise after saturation of the binding capacities of plasma proteins and by displacement of the bound xenobiotic by another chemical with higher affinity, which increases the free fraction of the formerly bound xenobiotic. This will result in an increased equilibrium concentration of the xenobiotic in plasma and in the target organ, with potentially harmful consequences.

2.4. Storage of Xenobiotics in Organs and Tissues

Absorbed xenobiotics may be concentrated in specific organs or tissues. The concentration of a xenobiotic in a specific tissue may cause toxic effects to that particular tissue; some xenobiotics actually attain their highest concentration at the site of toxic action. However, other xenobiotics may be concentrated in tissues without harmful consequences. Some tissues have a high capacity to accommodate certain xenobiotics and may release them only slowly. The compartment or tissue in which a chemical is concentrated can also be considered as a storage depot for this xenobiotic. If a chemical is stored in a depot and thus removed from the site of action (e.g., polychlorinated biphenyls in fat or lead in bone), no immediate manifestation of toxicity may be observed, even though a potential for adverse effects exists. For example, lead stored in bone does not cause a toxic response but has the potential for mobilization and thus for migration into soft tissues; toxic effects may appear after mobilization. As the xenobiotic in storage depots is in equilibrium with the free xenobiotic in plasma, mobilization is constant, and exposure of the target organ to low concentrations of the xenobiotic is constant. Some storage depots for specific chemicals follow:

Lead	bone
Fluoride	bone, teeth
Cadmium	kidney
Iron	transferrin (a blood protein)
Polychlorinated pesticides such as DDT	fat
Arsenic	skin

Liver, kidney, fat, bone, and plasma proteins may serve as storage depots for absorbed xenobiotics. Both liver and kidney have a high capacity to store xenobiotics and are major storage sites for a multitude of chemicals. Accumulation of circulating xenobiotics form the blood by active transport systems and binding to certain tissue constituents are major mechanisms involved in the renal and hepatic storage.

Several thiol-rich proteins present in liver and kidney have a high affinity for xenobiotics [28]. The binding protein ligandin, which as a glutathione *S*-transferase also has enzymatic activity (Section 2.5.5.6) and thus participates in xenobiotic biotransformation, binds organic acids, some azo dyes, and corticosteroids. Metallothionein, a cysteine-rich protein present in liver and kidney, serves as a binding and storage protein for metals such as cadmium and zinc. Its biosynthesis increases after exposure to metals,and this may result in storage of a considerable percentage of cumulative metal dose as metalothionein complex in liver and kidney.

Highly lipophilic chemicals rapidly penetrate membranes and are thus efficiently taken up by tissues. Lipophilic substances that are inefficiently biotransformed accumulate in the most lipophilic environment in the organism, fat. Most xenobiotics seem to accumulate by physical dissolution in neutral fats, which may constitute between 20 and 50% of the body weight in human males. Large amounts of lipophilic xenobiotics may therefore be present in fat; for xenobiotics that do not undergo biotransformation (e.g., 2,3,7,8-tetrachlorodibenzodioxin), determination of the concentration in body fat is a good measure for exposure.

Usually, xenobiotics stored in fat also do not induce toxic responses, since the xenobiotic is not readily available at the target site. However, during rapid mobilization of fat during disease or starvation, a sudden increase in the plasma concentration, and thus toxic effects in target organs,

may occur. For example, signs of organochlorine pesticide intoxication have been observed after starvation in animals pretreated with persistent organochlorine pesticides.

Some xenobiotics have a high affinity for bone and may accumulate in the bone matrix. For example, 90% of the lead and a major part of the strontium present in the body after chronic exposure are stored in the skeleton [29]. Lead and strontium accumulate in bone due to their similarity with calcium; inorganic fluoride, which is also a "bone-seeker", accumulates in bone due to similarities in size and charge to the hydroxyl ion. The storage of xenobiotics in bone may or may not be responsible for toxic effects. Lead stored in the skeleton is not toxic to bone, but both stored fluoride and stored strontium cause toxic effects to bone (fluorosis, osteosarcoma). Xenobiotics stored in bone are also in equilibrium with the unbound xenobiotic circulating in plasma and may thus be released.

Certain plasma proteins have a high affinity for xenobiotics, binding of a chemical to plasma proteins may constitute a both transport form and a storage form. Globulins such as transferrin (involved in iron transport) and ceruloplasmin (copper) and α- and β-lipoproteins (lipophilic xenobiotics and endogenous chemicals) may be involved in binding.

Storage in tissue may greatly alter the rate of excretion of a xenobiotic. Only xenobiotics present in plasma are available for distribution, biotransformation, and excretion. However, excretion or biotransformation alters the plasma concentration of the xenobiotic, and some of the stored chemical is released into plasma from the site of storage. Owing to this mechanism, the rate of excretion of a xenobiotic stored in tissues can be very low.

2.5. Biotransformation

Most xenobiotics entering the body are lipophilic. This property enables them to penetrate lipid membranes, to be transported by lipoproteins in the blood, and to be rapidly absorbed by the target organ. However, the efficient excretory mechanisms of the organism require solubility of the xenobiotic in aqueous media and thus a certain degree of hydrophilicity is required for efficient excretion. Lipophilic substances can only be excreted efficiently by exhalation, but this is restricted to volatile xenobiotics. In the absence of efficient means for excretion of nonvolatile chemicals, constant exposure or even intermittent single exposures to a lipophilic chemical could result in accumulation of the xenobiotic in the organism.

Therefore, animal organisms have developed a number of biochemical processes that convert lipophilic chemicals to hydrophilic chemicals and thus assist in their excretion. These enzymatic processes are termed biotransformation, and the enzymes catalyzing biotransformation reactions are referred to as biotransformationenzymes. The biotransformation enzymes differ from most other enzymes by having a broad substrate specifity and by catalyzing reactions at comparatively low rates. The low rates of biotransformation reactions are often compensated by high concentrations of biotransformation enzymes. For example, ca. 5 wt% of the protein in rat liver consist of cytochromes P450, which are major biotransformation enzymes.

The broad specifity of the biotransformation enzymes likely has evolutionary reasons. Biotransformation enzymes have evolved to facilitate the excretion of lipophilic chemicals present in the diet of animals. The broad substrate specificity helped to adjust to new dietary constituents and thus led to evolutionary advantages.

Biotransformation is generally the sum of several processes by which the structure of a chemical is changed during passage through the organism. The metabolites formed from the parent chemical are usually more water soluble; the increased water solubility reduces the ability of the metabolites to partition into membranes, restricts renal and intestinal reabsorption, and thus facilitates excretion with urine or bile.

2.5.1. Phase-I and Phase-II Reactions

Xenobiotic metabolism is catalyzed by a number of different enzymes. For solely operational purposes, the biotransformation enzymes are separated into two phases. In phase-I reactions, which involve oxidation, reduction, and hydrolysis, a polar group is added to the xenobiotic or is exposed by the biotransformation enzymes. Phase-II reactions are biosynthetic and link the metabolite formed by phase-I reactions to a polar

endogenous molecule to produce a conjugate. Various endogenous molecules with high polarity and are utilized for conjugation; the resulting conjugates are often ionized at physiological pH and thus highly water soluble. Moreover, the moieties used for conjugation are often recognized by specific active transport processes, which assist in their translocation across plasma membranes and thus further enhance the rate of excretion.

The fate of a particular chemical and the participation of the various phase-I and phase-II biotransformation enzymes is determined by its chemical structure; biotransformation is usually complex and often integrated. Many chemicals bearing functional groups undergo conjugation without prior phase-I biotransformation, whereas others are oxidized or reduced prior to conjugation. However, chemicals lacking functional groups may also undergo phase-II biotransformations without being subjected to a prior phase-I reaction (examples are 1,2-dibromoethane and perchloroethene; see Section 2.5.4).

2.5.2. Localization of the Biotransformation Enzymes

The biotransformation enzymes are localized mainly in the liver. A significant fraction of the blood from the splanchnic area, which also contains xenobiotics absorbed from the intestine, enters the liver. Therefore, the liver has developed the capacity to enzymatically modify most of these chemicals before storage, release, or excretion. However, most other tissues also have the capacity to catalyse biotransformation reactions; indeed, most tissues tested have shown the presence of enzymes which can catalyze biotransformation reactions. The contribution of extrahepatic organs to the biotransformation of a chemical depends on many factors, including chemical structure, dose, and route of administration. However, biotransformation of a chemical within an extrahepatic tissue may have toxic effects on this specific tissue and may thus have important toxicological consequences.

Inside cells, phase-I enzymes are mainly present in the endoplasmatic reticulum, a myriad of lipoprotein membranes extending from the mitochondria and the nucleus to the plasma membranes of the cell. When an organ is homogenized, the endoplasmatic reticulum is broken and membrane fragments are sealed of to form microvesicles. These microvesicles can be sedimented by differential centrifugation, and the materials thus obtained is known as a microsomes. They are highly enriched in vesicles from the endoplasmatic reticulum and retain active biotransformation enzymes. Microsomes are often used to study the enzymatic biotransformation of xenobiotics in vitro.

The presence of phase-I enzymes within membranes has important implications, since lipophilic chemicals will preferentially distribute into lipid membranes; thus, high concentrations of lipophilic xenobiotics are present at this site of biotransformation. In contrast to phase-I enzymes, phase-II enzymes are often soluble, non-membrane-associated, and present in the cytoplasm of the cell. They are found in the supernatant (cytosol) obtained by ultracentrifugation of homogenized tissues. The subcellular localizations of enzymes responsible for biotransformation afollow:

Phase-I enzymes	
–Cytochrome P450	microsomal
–Flavin-dependent mono-oxygenase	microsomal
–Prostaglandin synthase	microsomal
–Epoxide hydrolase	microsomal/cytosolic
Phase-II enzymes	
–UDP-glucuronyl-transferases	microsomal
–Sulfotransferases	cytosolic
–*N*-acetyltransferases	cytosolic
–Glutathione *S*-transferase	cytosolic/microsomal

2.5.3. Role of Biotransformation in Detoxication and Bioactivation

The general purpose of biotransformation reactions is detoxication, since xenobiotics should be transformed to metabolites which are more readily excreted. However, depending on the structure of the chemical and the enzyme catalyzing the biotransformation reaction, metabolites with a higher potential for toxicity than the parent compound are often formed. This process is termed bioactivation and is the basis for the toxicity and carcinogenicity of many xenobiotics with a low chemical reactivity (see Section 2.5.6). The interaction of the toxic metabolite initiates events that ultimately may result

in cell death, cancer, teratogenicity, organ failure, and other manifestations of toxicity. Formation of reactive and more toxic metabolites is more frequently associated with phase-I reactions; however, phase-II reactions and combinations of phase-I and phase-II reactions may also be involved in toxication.

2.5.4. Phase-I Enzymes and their Reactions

Phase-I reactions are catalyzed by microsomal monooxygenases and peroxidases, cytosolic and mitochondrial oxidases, reductases, and hydrolytic enzymes. All these reactions add or expose functional groups which can be conjugated later.

2.5.4.1. Microsomal Monooxygenases: Cytochrome P450

Microsomal monooxygenases are the cytochrome P450 enzymes and the mixed-function amine oxidase or flavin-dependent monooxygenase. Both enzyme systems add a hydroxyl moiety to the xenobiotic. Cytochrome P450, a carbon monoxide binding hemoprotein in microsomes, is the most important enzyme system involved in phase-I reactions. The name cytochrome P450 is a generic term applied to a group of hemoproteins defined by the unique spectral property observed when reduced cytochrome P450 (Fe^{2+}) is treated with carbon monoxide. The complex formed has a maximum absorption at 450 nm imparted by the presence of an axial thiolate ligand on the heme iron atom. This spectral characteristic is only present when the protein is intact and catalytically functional. Denatured cytochrome P450 shows, like other heme proteins, an absorbance maximum at 420 nm.

Cytochrome P450 enzymes are a coupled enzyme system composed of the heme-containing cytochrome P450 and the NADPH-containing cytochrome P450 reductase [30]. This flavoprotein has a preference for NADPH as its cofactor and transfers one or two electrons from NADPH to cytochrome P450. Cytochrome P450 and the reductase are embedded into the phospholipid matrix of the endoplasmatic reticulum. The phospholipid matrix is crucial for enzymatic activity since it facilitates the interaction between the two enzymes. The importance of the phospholipid matrix is indicated by the following: Both cytochrome P450 and cytochrome P450 reductase can be purified to apparent homogeneity; the enzymatic activity of the purified and recombined enzymes is dependent on the addition of phospholipids.

In vertebrates, the highest concentrations of cytochrome P450 are found in the liver, but cytochrome P450 enzymes are also present in lung [31–35], kidney, testes, skin, and gastrointestinal tract [36]. The presence of several forms of cytochrome P450 with different substrate specificity and different rates of biotransformation for certain xenobiotics was indicated by studies in the 1970s. In the early 1980s, several different cytochrome P450 enzymes from rodents were purified to apparent homogenicity. Moreover, a large number of cytochrome P450 enzymes have been purified from human organs. All these cytochrome P450 enzymes share the heme, but they differ in both the composition and thus the structure of the polypeptide chain and in the reactions they catalyze [37–40].

The individual enzymes are regulated in their expression by a variety of factors such as treatment with xenobiotics, species, organ, sex, and diet. Because of the multitude of enzymes present, the term “superfamily” of cytochromes P450 is frequently used. In mammals, two general classes of cytochrome P450 exist: six families involved in steroid metabolism and bile acid biosynthesis, and four families containing numerous individual cytochromes P450, mainly responsible for xenobiotic biotransformation. A complex nomenclature, based on amino acid sequence similarity, has been developed to designate individual cytochromes P450. The genes for the individual enzymes are named by the root CYP followed by a number designating the family, a letter for the subfamily, and another number denoting the individual enzyme (see Table 9).

Table 9. Mammalian cytochromes P450 involved in xenobiotic biotransformation

Family	Number of subfamilies	Number of forms
CYP1	1	2
CYP2	8	59
CYP3	2	11
CYP4	2	10

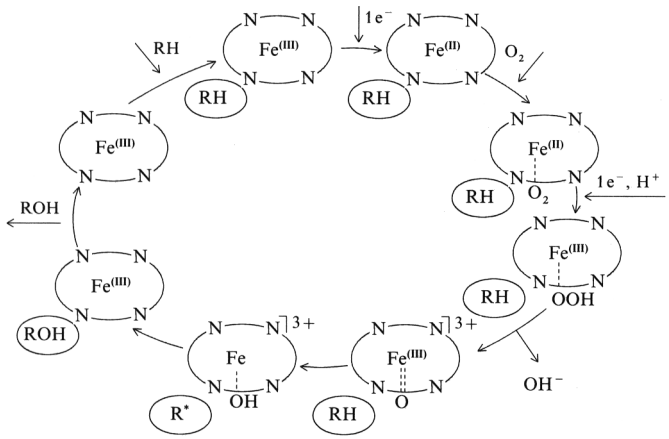


Figure 18. Mechanisms of electron transfer and xenobiotic oxidation by cytochrome P450

Table 10. Oxidations catalyzed by cytochrome P450

Type of reaction	Examples
Aliphatic hydroxylation	$R-CH_2-CH_2-CH_3 \xrightarrow[P450]{O_2} R-CH_2-CHOH-CH_3$
N-Dealkylation	$\begin{matrix}R\\ \diagdown\\ N-CH_3\\ \diagup\\ R^1\end{matrix} \xrightarrow{P450} \left[\begin{matrix}R\\ \diagdown\\ N-CH_2-OH\\ \diagup\\ R^1\end{matrix}\right] \longrightarrow \begin{matrix}R\\ \diagdown\\ NH\\ \diagup\\ R^1\end{matrix} + \begin{matrix}H\\ \diagdown\\ C=O\\ \diagup\\ H\end{matrix}$
O-Dealkylation	$O_2N-C_6H_4-OCH_3 \longrightarrow [-O-CH_2-OH] \xrightarrow{P450} O_2N-C_6H_4-OH + \begin{matrix}H\\ \diagdown\\ C=O\\ \diagup\\ H\end{matrix}$
Epoxidation	$H_2C=CH_2 \xrightarrow{P450}$ 

Reactions catalyzed by cytochrome P450. Cytochromes P450 are monooxygenases. They utilize one of the oxygen atoms of molecular oxygen and incorporate it into the xenobiotic RH:

$$RH+O_2+NADPH+H^+ \rightarrow ROH+H_2O+NADP^+$$

The other oxygen atom is reduced to water with consumption of NADPH as reducing cofactor. The likely mechanisms of electron transfer and xenobiotic oxidation is shown in Figure 18.

In the first step of the catalytic cycle, the xenobiotic combines with the oxidized form of cytochrome P450 (Fe^{3+}) followed by a one-electron reduction by NADPH-cytochrome P450 reductase to form a reduced (Fe^{2+}) cytochrome P450-substrate complex. This complex then combines with molecular oxygen, and another electron from NADPH is accepted. In a series of further steps, which are not completely understood, an oxygen atom from the intermediate is transferred to the substrate, while the other oxygen atom is reduced to water. In the last step of the catalytic cycle, the oxidized substrate dissociates and regenerates the oxidized form of cytochrome P450. Examples for oxidation reactions catalyzed by cytochromes P450 are shown in Table 10.

Cytochromes P450 may catalyze the hydroxylation of carbon–hydrogen bonds to transform hydrocarbons to the corresponding alcohols. In larger aliphatic chains, the $(\omega - 1)$ position is often a favored point of attack. Oxidative *N*-, *O*-, or *S*-dealkylation and oxidative dehalogenation are similar in mechanism to aliphatic hydroxylation, but, due to further reactions of the intermediate products, give different end products. Olefins are also oxidized by cytochrome P450, and with some substrates, epoxides are

formed as products. The reaction, however, does not proceed in a concerted manner, but involves discrete ionic intermediates, which may also rearrange to products other than epoxides, as shown for chloroolefins in Figure 19.

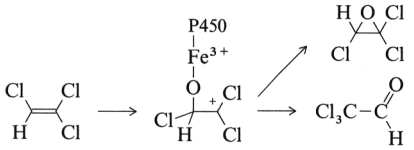


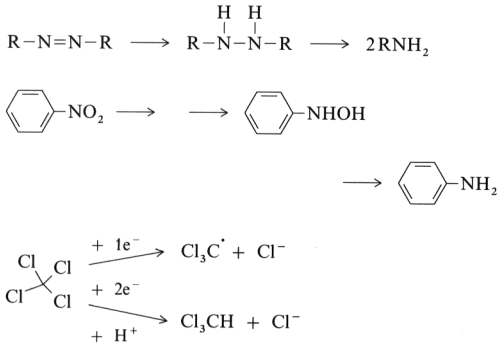
Figure 19. Mechanism of oxidation and rearrangement of trichloroethylene to chloral and trichlorooxirane, respectively

Oxidation at sulfur or nitrogen occurs by the addition of oxygen at the unshared electron pair on the sulfur or nitrogen atom. The products formed (sulfoxides or hydroxylamines) may be stable (many sulfoxides), may be further oxidized by other enzymes in the organism (e.g., hydroxylamines), or may decompose to sulfur and the corresponding oxo compound.

The above-mentioned reactions may be catalyzed by most cytochromes P450 involved in xenobiotic biotransformation; the type of reaction catalyzed seems to be more influenced by steric factors regarding the substrate-binding site of individual cytochromes P450 than by electronic factors. As shown in Table 11, which gives an overview of human cytochrome P450s involved in the biotransformation of xenobiotics and drugs, steric factors are likely major determinants of the substrate specificity of cytochrome P450 enzymes.

In addition to promoting oxidative metabolism, cytochrome P450 may also catalyze reductive biotransformation reactions [41, 42]. These reaction are favored under reduced oxygen pressure or occur with xenobiotics lacking oxidizable C–H bonds or olefinic moieties. In these cases,instead of oxygen, the xenobiotic accepts one or two electrons from NADPH-cytochrome P450 reductase or from cytochrome P450. Reductive biotransformation catalyzed by cytochrome P450 has been demonstrated with some azo dyes and several aromatic nitro compounds. The double bond in azo compounds may be progressively reduced to give amine metabolites; aromatic nitro groups may also be reduced via the nitrone and the hydroxylamine to the corresponding amine.

The reductive biotransformation of polyhalogenated alkanes is exemplified by the one-electron reduction of carbon tetrachloride to the trichloromethyl radical and chloride; reductive biotransformation of carbon tetrachloride by a two-electron reduction results in formation of chloroform:



2.5.4.2. Microsomal Monooxygenases: Flavin-Dependent Monooxygenases

Tertiary amines and sulfur-containing drugs have been known to be metabolized to *N*-oxides or sulfoxides by a microsomal monooxygenase which is not dependent on cytochrome P450 [43, 44]. This enzyme, which is historically referred to as mixed-function amine oxidase, is a flavoprotein that is present in the endoplasmatic reticulum. It is capable of oxidizing nucleophilic nitrogen and sulfur atoms in xenobiotics. However, this enzyme shows a catalytic mechanism different from those of other heme- or flavin-containing enzymes. Like other monooxygenases, flavin-containing monooxygenases require molecular oxygen and NADPH as cofactors for oxygenation. In contrast to the other monooxygenases, flavin-dependent monooxygenases do not contain heme or iron, and the binding of the substrate is not required for the generation of the enzyme bound oxygenating intermediate (Fig. 20).

The active, oxygenating form of the enzyme is present in the cell, and any soft, oxidizable nucleophile that can gain access to the enzyme-bound oxygenating intermediate will be oxidized. Precise fit of substrate to the enzyme is not necessary. This property seems to be largely responsible for the broad substrate specificity of flavin-dependent monooxygenases. Flavin-

Table 11. Human cytochromes P450 identified as major catalysts in the biotransformation of specific xenobiotics that seem to play major roles in the oxidation of substrates listed

Cytochrome P450			
1A1	1A2	2E1	3A4
Benzo[*a*]pyrene	phenacetin	vinyl chloride	aflatoxin B_1
Other polycyclic hydrocarbons	1-aminofluorene	trichloroethylene	17-β-estradiol
	2-amino-3-methylimidazo-[4,5-*f*]quinoline	halothane benzene	6-aminochrysene sterigmatocystine
	2-naphthylamine	dimethylnitrosamine acetaminophen	nifedipine ethinylestradiol

dependent monooxygenase catalyzes the oxidation of a wide variety of xenobiotics with few, if any, common structural features at maximum rate (Table 12).

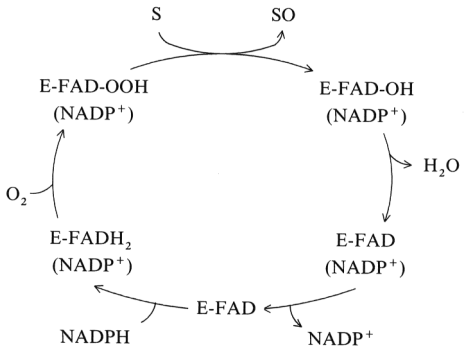
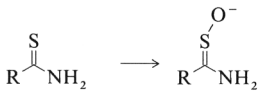


Figure 20. Mechanism of xenobiotic oxidation by the flavin-dependent monooxygenase
FAD = flavineadenine dinucleotide

Many essential xenobiotics also bear functionalities that are oxidized by flavin-dependent monooxygenases. However, these enzymes apparently discriminate between physiologically essential and xenobiotic soft nucleophiles and seem to exclude the former.

As with cytochrome P450, species- and tissue-specific forms of flavin-dependent monooxygenase have been described. Species differences in hepatic flavin-dependent monooxygenase seem to be quantitative rather than qualitative, whereas tissue specific forms in the same species are clearly distinct enzymes. For example, hepatic and pulmonary flavin-dependent monooxygenases in rabbits exhibit distinct, but overlapping substrate specificities and are different gene products. Recent studies have shown that several isoforms of flavin-dependent monooxygenase exist; all isoforms show differences in their distribution in species and in organs within species [45].

Table 12. Oxidations catalyzed by flavin-dependent monooxygenases

Type	Examples
Amine oxidation	
Hydroxylamine oxidation	
Thioamide oxidation	
Thiol oxidation	$2R-SH \longrightarrow R-S-S-R$
Disulfide oxidation	$R-S-S-R \longrightarrow 2R-SO_2$

The regulation of expression of flavin-dependent monooxygenase seems to be complex. Xenobiotics which were shown to increase the concentration of cytochrome P450 in mammals did not influence flavin-dependent monooxygenase concentrations. Recent evidence suggest that soft nitrogen and sulfur nucleophiles in the diet may act as inducers of flavin-dependent monooxygenases. Since the dietary inducers are continously taken up, flavin-dependent monooxygenases are present in maximal concentrations in rats on commercial rat chow.

2.5.4.3. Peroxidative Biotransformation: Prostaglandin-synthase

During the biosynthesis of prostaglandins, a polyunsaturated fatty acid, arachidonic acid, is oxidized to yield prostaglandin G_2, a hydroperoxy endoperoxide. This is further transformed to prostaglandin H_2. Both the formation of prostaglandin G_2 and its further transformation into prostaglandin H_2 are catalyzed by the same

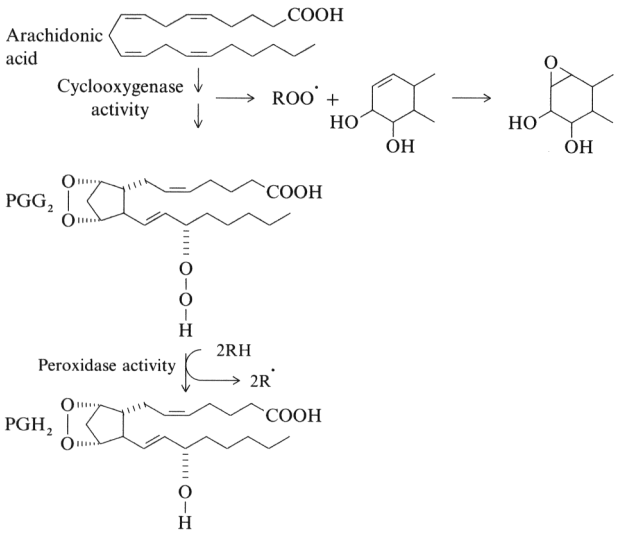


Figure 21. Cooxidation of xenobiotics during the biosynthesis of prostaglandins

enzyme, prostaglandin synthase [46–48]. This enzyme is a glycoprotein with a molecular mass of approximately 70 000 Dalton and contains one heme per subunit. The enzyme is found in high concentrations in germinal vesicles and renal medulla, but also in several other tissues such as skin and adrenals [49]. The cyclooxygenase and peroxidase activity of prostaglandin synthase generate enzyme- and substrate-derived free-radical intermediates (Fig. 21).

Biotransformation of xenobiotics may be associated both with the cyclooxygenase and the peroxidase activity of prostaglandin synthase (Fig. 21). During the cyclooxygenase-catalyzed conversion of arachidonic acid to prostaglandin G_2 peroxy radicals are formed as intermediates. These lipid peroxy radicals represent a source of reactive oxygen metabolites (see below) and can in turn biotransform xenobiotics. Oxidation by the cyclooxygenase activity of prostaglandin synthase is important in the oxidation of diols derived from carcinogenic polycyclic aromatic hydrocarbons and transforms these diols to diol epoxides (Fig. 21, top).

During the reduction of the peroxide prostaglandin G_2 to prostaglandin H_2, the peroxidase undergoes a two-electron oxidation. To return to the ground state the enzyme requires two consecutive one-electron reductions, which are achieved by abstracting electrons from available donors. In addition to endogenous substrates, xenobiotics may act as electron donors and may thus be oxidized to radicals (Fig. 21, bottom). This process is termed cooxidation of xenobiotics. Classes of xenobiotics that undergo cooxidation during prostaglandin syntheses are aromatic amines, phenols, hydroquinones, and aminophenols. The role of prostaglandin synthase in the biotransformation of xenobiotics is somewhat unclear, since many of the end products of prostaglandin-synthase-mediated cooxidation of xenobiotic are identical to those formed by cytochrome P450. Therefore, it is assumed that prostaglandin synthase may contribute to the oxidative biotransformation of xenobiotics in tissues low in monooxygenase activity [50].

In addition to prostaglandin synthase, other peroxidases may also participate in the oxidation of xenobiotics [51–53]. For example, mammary gland epithelium contains lactoperoxidase and leucocytes contains myeloperoxidase. The general reaction catalyzed by this enzymes involves the reduction of hydroperoxide coupled to the oxidation of the substrate:

$$ROOH + XH \rightarrow ROH + XOH$$

The availability of peroxides in tissues likely controls the extent of peroxidative biotransformation; however, the availability of hydrogen peroxide is usually low due to efficient

scavenging by catalase and glutathione peroxidase. Therefore, peroxidative metabolism occurs mainly in tissues which can maintain an oxidizing environment.

2.5.4.4. Nonmicrosomal Oxidations

Several enzymes located in mitochondria or the cytoplasm of the cell may also catalyze the oxidation of xenobiotics. In contrast to cytochromes P450 with their broad substrate specificity, most of the nonmicrosomal oxidases have a more narrow substrate specificity and accept only xenobiotics bearing specific functional groups as substrates. Alcohol dehydrogenases catalyze the oxidation of alcohols to aldehydes or ketones:

$$RCH_2OH + NAD^+ \rightarrow RCHO + NADH + H^+$$

The enzyme is mainly found in the soluble fraction of liver, but also in other organs such as the kidney and lung and is responsible for the oxidation of ethanol. The expression of alcohol dehydrogenase is under genetic control, which gives rise to a number of variants with differing activities. Usually, the oxidation of alcohols to aldehydes is reversible, since the reduction of aldehydes is also efficiently catalyzed by aldehyde reductases. However, in vivo, the reaction proceeds in the direction of alcohol consumption since aldehydes are further oxidized by aldehyde dehydrogenases. These enzymes catalyze the formation of acids from aliphatic and aromatic aldehydes:

$$RCHO + NAD^+ \rightarrow RCOOH + NADH + H^+$$

The reaction may be catalyzed by aldehyde dehydrogenase, which has broad substrate specificity, and several isozymes of aldehyde dehydrogenase are found in liver cytosol, mitochondria, and microsomes with characteristic substrate specificities. Other enzymes in the soluble fraction of liver that can oxidize aldehydes are the flavoproteins aldehyde oxidase and xanthine oxidase.

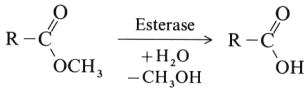
Monoamine oxidases are a family of flavoproteins present in many tissues including liver, kidney, brain, and intestine. These mitochondrial enzymes have a broad and overlapping substrate specificity and oxidize a varietyof amines:

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O$$

The monoamine oxidase found in the central nervous system is concerned primarily with neurotransmitter turnover.

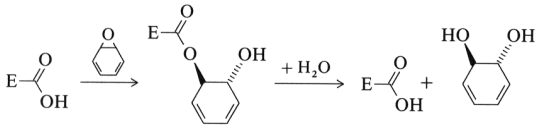
2.5.4.5. Hydrolytic Enzymes in Phase-I Biotransformation Reactions

Many tissues contain enzymes with carboxylesterase and amidase activity. These enzymes are located both in microsomal and soluble fraction and hydrolyse ester and amide linkages in xenobiotics.



Although esterases and amidases were thought to be different enzymes, all purified esterases have been demonstrated to have amidase activity; similarly, all amidases have esterase activity. In general, esters are cleaved more rapidly than amides. The expression of many esterases is under genetic control; thus, extremes of high/low esterase activity and resistance/sensitivity to toxic effects mediated by esterases are known.

Epoxide hydrolase is an important enzyme cleaving aliphatic and aromatic epoxides. The enzyme hydrates arene oxides and aliphatic epoxides to the corresponding *trans*-1,2-diols [54]. Water is required as cofactor, and the catalytic mechanism of epoxide hydrolases involves ester formation of the oxirane with a carboxylic acid function at the active site of the enzyme and hydrolysis of this ester by water; no metals or other cofactors are required.



Microsomal epoxide hydrolases are thought to be present in close proximity to the microsomal cytochromes P450; in most cases, the conversion of the epoxide to the less reactive diol is

considered to represent an important detoxication reaction for metabolically formed oxiranes. Epoxide hydrolases are found in many tissues such as liver, kidney, testes, and intestine. Their distribution is heterogenous between different cell types in a specific organ; in addition, several forms of microsomal epoxide hydrolases with broad substrate specificity have been found in different animal species. Moreover, in animals, in addition to membrane-bound epoxide hydrolase, a soluble epoxide hydrolase is present in cytoplasm of several tissues [55].

2.5.5. Phase-II Biotransformation Enzymes and their Reactions

Products of the phase-I biotransformation reactions carrying functional groups such as hydroxyl, amino, or carboxyl often undergo a conjugation reaction with an endogenous substrate. The endogenous substrates may include sugar derivatives, sulfate, amino acids, and small peptides (glutathione). The conjugation products are usually more polar and thus more readily excreted than their parent compounds.

In contrast to phase-I biotransformation reactions, phase-II reactions are biosynthetic and require energy to drive the reaction. Energy is usually consumed to generate a high-energy cofactor or an activated intermediate and then utilized as cosubstrate. Thus, depletion of the cofactor or general interference with cellular energy status may interfere with the ability of cells to conduct phase-II biotransformation reactions.

2.5.5.1. UDP-Glucuronyl Transferases

Glucuronidation represents one or the main phase-II biotransformation reactions in the conversion of both endogenous and exogenous compounds to water soluble products [56 – 59]. The formed glucuronides are excreted with bile or urine. The formation of an activated glucuronide (uridine diphosphate glucuronic acid, UDPGA), is required for glucuronide formation. UDPGA is formed in a sequential reaction from uridine and glucose-1-phosphate.

$$\text{Uridine triphosphate} + \text{Glucose 1-phosphate} \xrightarrow[\text{Pyrophosphorylase}]{\text{UDPG}} \text{Uridine diphosphate glucose} + \text{Pyrophosphate}$$

$$\text{Uridine diphosphate glucose} + 2\,NAD^+ + H_2O \xrightarrow{\text{Dehydrogenase}} UDPGA + 2\,NADH_2$$

The enzymes that carry out the coupling of the xenobiotic with UDPGA are termed UDP-glucuronyl transferases. They couple D-glucuronic acid with a wide variety of xenobiotics carrying functional groups to give β-D-glucuronides [57]. These glucuronides are highly polar and ionized at physiological pH, and hence are rapidly excreted. The membrane-bound UDP-glucuronyl transferases are found in highest concentration in the liver, but also in most other tissues studied. The reaction catalyzed involves a nucleophilic displacement (S_N2) of the functional group of the substrate with Walden inversion (Fig. 22).

UDP-glucuronyl transferases, like cytochrome P450, represent a family of enzymes, and at least ten individual forms are known. The various forms respond differently to inducers and have preferences for certain classes of chemicals. UDP-glucuronyl transferases catalyze the conjugation of numerous functional groups of xenobiotics with glucuronic acids. Some typical examples are shown in Table 13.

Glucuronides formed in the liver are excreted with urine or bile. Aglycones with molecular masses higher than 300 are transformed to glucuronides that surpass the molecular mass

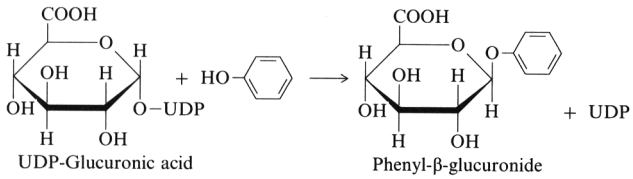
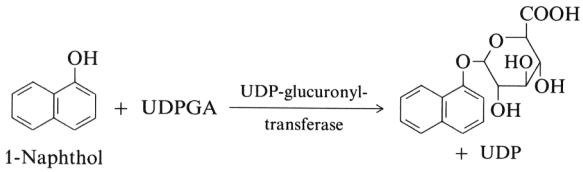
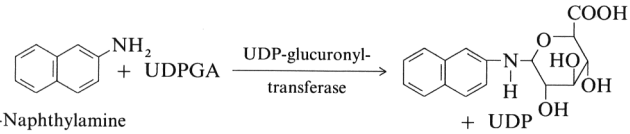
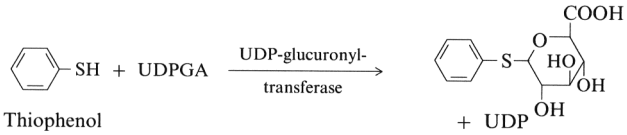


Figure 22. Conjugation of phenol to phenyl glucuronide catalyzed by UDP-glucuronyltransferase (UDP = uridine diphosphate)

Table 13. Typical conjugation reactions catalyzed by UDP-glucuronyltransferases

Type of reaction	Examples
O-Glucuronide formation	 1-Naphthol
N-Glucuronide formation	 2-Naphthylamine
S-Glucuronide formation	 Thiophenol

threshold for biliary excretion (see Section 2.5.8.2) and will thus be excreted with bile into the intestine. Glucuronides may by cleaved there by β-glucuronidase present in intestinal microflora to the respective aglycone, which may be reabsorbed from the intestine and translocated back to the liver with the blood. The resulting cycle is called enterohepatic circulation; compounds that undergo enterohepatic circulation are only slowly excreted and usually have a longer half-life in the body.

2.5.5.2. Sulfate Conjugation

The formation of water-soluble sulfate esters is observed with many xenobiotics carrying functional groups such as alcohols, phenols, and arylamines. These reactions are catalyzed by sulfotransferases, a large group of soluble enzymes found in many tissues [60–62]. Sulfotransferases catalyze the transfer of a sulfate group from the "active sulfate" 3′-phosphoadenosine-5′-phosphosulfate to hydroxyl groups and amines. The resulting products are referred as sulfate esters of sulfamates.

The products of this reaction are ionized at physiological pH and may therefore be rapidly excreted in urine; thus, sulfate conjugation is an effective mechanism to enhance the rate of excretion of many xenobiotics. Sulfotransferases are also a family of enzymes, and at least four different classes of sulfotransferases are involved in biotransformation reactions: sulfotransferases, hydroxysteroid sulfotransferases, estrone sulfotransferases, and bile salt sulfotransferases; each class again has been divided into several distinct forms differing in substrate specificity, optimum pH and immunological properties.

Sulfate conjugation also requires a sulfate donor, 3′-phospho-adenosine-5′-phosphosulfate. 3′-Phospho-adenosine-5′-phosphosulfate is likely synthesized in the cytosol of most mammalian cells by a two-step reaction consuming ATP and utilizing inorganic sulfate originating from the catabolism of cysteine or from diet. In the first step of this sequence the sulfation of ATP to adenosine 5′-phosphosulfate is catalyzed by an ATP sulfurylase. Adenosine 5′-phosphosulfate is further transformed to 3′-phosphoadenosine 5′-phosphosulfate by an adenosine 5′-phosphosulfate kinase. However, the equilibrium concentration of 3′-phosphoadenosine 5′-phosphosulfate in mammalian cells may be low, and due to the tight coupling of two enzymes in 3′-phosphoadenosine 5′-phosphosulfate, biosynthesis may proceed rapidly.

$$SO_4^{2-} + ATP \xrightarrow{\text{Sulfurylase}} APS + \text{Pyrophosphate}$$

$$APS + ATP \xrightarrow{\text{APS-phosphokinase}} PAPS + ADP$$

Despite the rapid synthesis, the sulfation of xenobiotics may by limited by reduced availability of 3′-phosphoadenosine 5′-phosphosulfate. The availability of the cofactor of the synthesis reaction, sulfate, may be limited by consumption due to sulfation or the limited availability of free cysteine for transformation to sulfate. Therefore, the sulfation capacity for certain xenobiotics is dependent on dose. Following administration of low doses the compound may be excreted as sulfate; after high doses, the capacity of sulfate conjugation may be saturated, and other biotransformation reactions such as glucuronide formation may become more important for biotransformation.

2.5.5.3. Methyl Transferases

A large number of alcohols, phenols, amines, and thiols present both as endogenous and exogenous compounds may be methylated by several *N*-, *O*- and *S*-methyl transferases. The most common donor for the methyl group is *S*-adenosyl methionine, which is formed from methionine and ATP. Often, these reactions do not increase the water solubility of a xenobiotic, but they are regarded as phase-II reactions since they mask potentially toxic functional groups and thus may serve as detoxication reactions.

A large variety of enzymes catalyze methylations of xenobiotics. The more important enzymes involved in methylation reactions are usually found in many tissues and are present in the soluble fraction of tissues. Some of the enzymes have a high specificity for certain endogenous compounds such as histamine or noradrenaline; others, such as catechol *O*-methyl transferase, metabolize both endogenous catechols and certain xenobiotics carrying aromatic rings with catechol functionalities [63].

2.5.5.4. *N*-Acetyl Transferases

Aromatic amines, hydrazines, sulfonamides, and certain aliphatic amines are biotransformed into amides in a reaction catalyzed by *N*-acetyl transferases. Enzymes that catalyze the acetylation of amines are designated as acetyl CoA: amine *N*-acetyl transferases. These enzymes utilize acetyl coenzyme A as cofactor [64]. The acetylation reaction of arylamines occurs in discrete steps. In the first step, the acetyl group from acetyl coenzyme A is transferred to the *N*-acetyl transferase, which then acetylates the arylamine, thus regenerating the enzyme and forming the amide (Fig. 23).

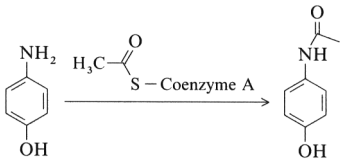


Figure 23. Mechanism of the *N*-acetylation of amines by *N*-acetyl transferase

N-Acetyl transferases are found in a number of different forms in cytosol of many tissues. In many species, the expression of *N*-acetyl transferases is under genetic control, and polymorphism for the expression of *N*-acetyl transferase has been found in several animal species and in humans. The transfer of an acetyl group to amines is reversible, and deacetylation of amides occurs in many species; as noted above, there are large differences between strains, species, and individuals in the extent of expression of amidases [65].

2.5.5.5. Amino Acid Conjugation

Exogenous carboxylic acids are conjugated with a variety of amino acids to form amides. Substrates for conjugation are mainly carboxylic acids containing aromatic rings. Glutamate and glycine appear to be the most common amino acids involved in these conjugation reactions in mammals; in other species such as reptiles and birds, ornithine is involved. The reaction proceeds in two steps and is catalyzed to two different enzymes. In the first step, the carboxylic acid is activated to form a coenzyme A derivative in a reaction involving coenzyme A and ATP. The enzymes that catalyze this reaction are called ATP-dependent acid coenzyme A ligases and are present in mitochondria. They appear to be identical to the intermediate-chain-

length fatty acid:coenzyme A synthetase. The thus-formed coenzyme A thioester then transfers its acyl group to the amino group of the acceptor amino acid. This reaction is catalyzed by an *N*-acetyl transferase (Fig. 24). The enzymes catalyzing both steps in amino acid conjugation exist in several forms with different substrate specifities.

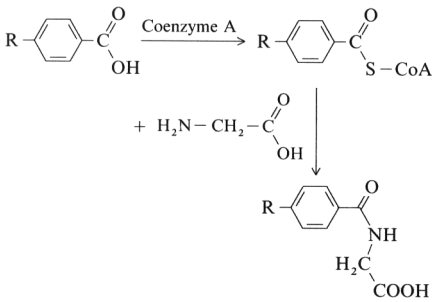


Figure 24. Amino acid conjugation of a xenobiotic carboxylic acid by ATP-dependent acid- coenzyme A ligases followed by *N*-acetyltransferase

2.5.5.6. Glutathione Conjugation of Xenobiotics and Mercapturic Acid Excretion

The conjugation of xenobiotics or their metabolites with the tripeptide glutathione is an important conjugation reaction. Glutathione is composed of the amino acids cysteine, glutamic acid, and glycine (γ-glutamylcysteinylglycine) and is present in many cells in high concentrations (up to 10 mM in liver cells) [66]. Since glutathione conjugation captures reactive electrophiles and transforms them into stable, often nontoxic thioethers, the formation of glutathione conjugates protects cells from the harmful effects of these electrophiles and thus serves as a major detoxication reaction (Section 2.5.6.5). Glutathione conjugation is catalyzed by a family of enzymes termed glutathione *S*-transferases, which are present in the highest concentration in the liver, but are also found in high activity in the kidney, testes, and lung [67]. Glutathione *S*-transferases exist in both membrane-bound and soluble forms; with most substrates, the activity of soluble glutathione *S*-transferase is higher than that of microsomal glutathione *S*-transferase [68].

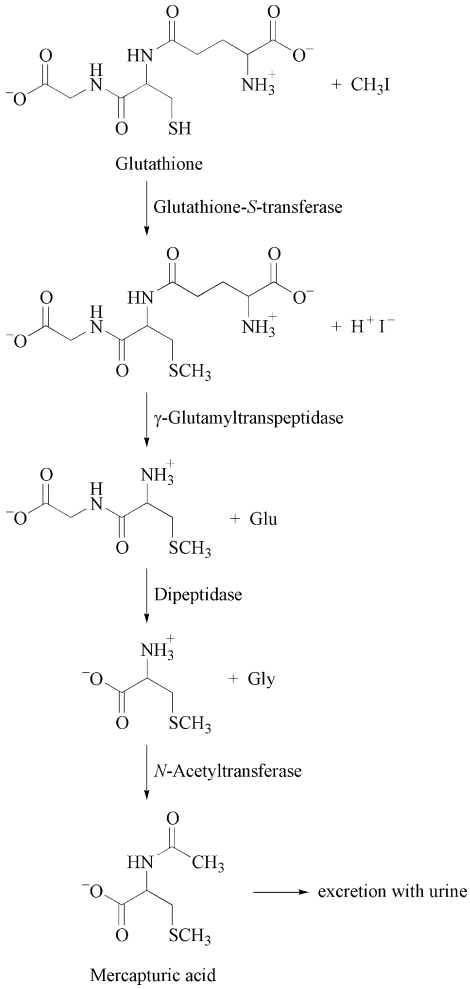
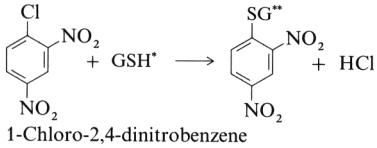
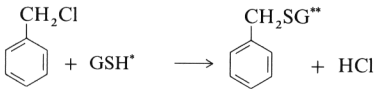
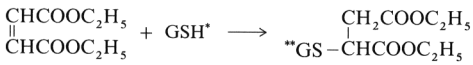
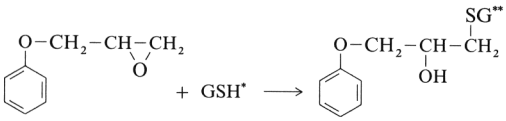


Figure 25. Formation of mercapturic acids by processing of glutathione *S*-conjugates as exemplified by the metabolism of methyl iodide

Cytosolic glutathione *S*-transferases exist in numerous different isoforms, each species being a dimer differing in subunit composition [67, 69]. The glutathione *S*-transferase gene family exists of at least six different families. In contrast to the multiple forms of soluble glutathione *S*-transferases thus possible, only one form of the membrane-bound enzyme is known. The glutathione *S*-transferases catalyze the reaction of the sulfhydryl group of glutathione with chemicals containing electrophilic carbon atoms (Table 14).

Thioethers are formed by reaction of the thiolate anion of glutathione with the electrophile; a spontaneous reaction, albeit at low rates, of

Table 14. Substrates for mammalian glutathione S-transferases

Type of reaction	Examples
Aryltransferase	 1-Chloro-2,4-dinitrobenzene
Arylalkyl-transferase	
Alkylene-transferase	
Epoxide-transferase	

GSH = Glutathione. ** GS = Glutathionyl residue.

the electrophile with glutathione without assistance by glutathione *S*-transferases is required for enzymatic catalysis. Glutathione thioethers formed in the organism are not excreted, but further processed to excretable mercapturic acids. Mercapturic acids are thioethers derived from *N*-acetyl-L-cysteine. Mercapturic acid formation is initiated by conjugation of the xenobiotic or an electrophilic metabolite with glutathione (Fig. 25).

This is followed by transfer of the glutamate by γ-glutamyltranspeptidase, an enzyme specifically recognizing γ-glutamyl peptides and found in high concentrations in the kidney and other excretory organs. Dipeptidases catalyze the loss of glycine from the intermediary cysteinylglycine *S*-conjugate to give the cysteine *S*-conjugate which, in the final step of mercapturic acid formation, is *N*-acetylated by a cysteine-conjugate-specific *N*-acetyltransferase using acetyl coenzyme A as cofactor. The mercapturic acids formed are readily excreted into urine by active transport mechanisms in the kidney [70].

Glutathione conjugation is one of the most important detoxication reactions for reactive intermediates formed in organisms. Usually, metabolically formed intermediates are efficiently detoxified, but under specific circumstances, glutathione conjugation may be overwhelmed by high concentrations of electrophiles, which result in covalent binding of intermediates to cellular macromolecules, disruption of important cellular functions, and cell death and necrosis (see Section 2.5.4.5).

2.5.6. Bioactivation of Xenobiotics

Many xenobiotics with low chemical reactivity (e.g., the solvent carbon tetrachloride, the environmental contaminant hexachlorobutadiene, and the heat-exchanger fluid tri-*o*-cresyl phosphate) cause toxic effects. These toxic effects are initiated by covalent binding to macromolecules of metabolites formed in the organism by biotransformation enzymes. This process is termed bioactivation. With many chemicals, reactive metabolites formed during bioactivation may be efficiently detoxified; thus, toxic effects only occur when the balance between the production of reactive metabolites and their detoxication is disrupted. For example, toxic effects may be observed with a certain chemical only when the formation of reactive intermediates is enhanced or when the capacity for detoxication is diminished.

Table 15. Basic mechanisms involved in the bioactivation of xenobiotics based on chemical reactivity of intermediates formed

Mechanism	Structure and reactivity of the intermediate	Examples
Biotransformation to stable but toxic metabolites	different structures, selective interaction of formed metabolite with specific acceptors, or disruption of specific biochemical pathways	dichloromethane, acetonitrile, parathion
Biotransformation to electrophiles	reactive electrophiles	dimethylnitrosamine, acetaminophen, bromobenzene
Biotransformation to free radicals	radicals	carbon tetrachloride
Formation of reactive oxygen metabolites	radicals	paraquat, aromatic nitro compounds

The mechanisms of bioactivation of xenobiotics may be classified into four categories describing the basic types of reactive intermediates formed and their potential reactivity (Table 15) [71–73].

2.5.6.1. Formation of Stable but Toxic Metabolites

This mechanisms is limited to a few selected chemicals because few xenobiotic metabolites are both stable and toxic. The bioactivation of the solvents *n*-hexane and dichloromethane are examples of this mechanism. *n*-Hexane produces a characteristic neuropathy and peripheral nerve injury after chronic exposure. The same typical manifestations of toxicity are also observed when the *n*-hexane metabolites 2-hexanone and 2,5-hexanedione are administered to animals. The mechanism of *n*-hexane neuropathy thus involves oxidation of *n*-hexane by cytochromes P450 at both ends of the carbon chain (ω–1 hydroxylation) and further oxidation of the thus-introduced alcoholic function. The 2,5-hexanedione formed reacts with critical lysine residues in axonal proteins by Schiff base formation followed by cyclization to give pyrroles [74]. Oxidation of the pyrrole residues then causes crosslinking between two *n*-hexane-modified proteins; the resulting changes in the three-dimensional structures of proteins perturb axonal transport and function and cause damage to nerve cells (Fig. 26).

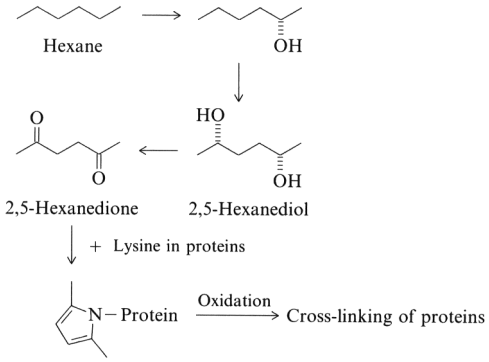
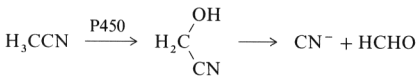
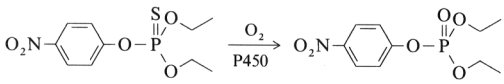


Figure 26. Bioactivation of hexane by cytochrome P450 to 2,5-hexanedione
Hexanedione reacts with lysine groups in proteins to form pyrroles; oxidation of two neighboring pyrrole residues causes the cross-linking of proteins.

Carboxyhemoglobin formation is observed after human exposure to dichloromethane. Dihalomethanes are oxidized by cytochrome P450, likely by P450 2E1, to carbon monoxide, which, due to its high affinity for iron(II)-containing porphyrins, binds to hemoglobin and interferes with oxygen transport in the blood. Other examples for the formation of stable, but toxic metabolites include the oxidation of acetonitrile to cyanide



and the oxidative desulfuration of parathion.



Parathion is a potent insecticide acting as an inhibitor of cholinesterase, but itself is only a weak cholinesterase inhibitor. Biotransformation of parathion by oxidative desulfuration to give the potent cholinesterase inhibitor paraoxone is responsible for the high insecticidal potency.

2.5.6.2. Biotransformation to Reactive Electrophiles

Biotransformation to reactive electrophiles is the most common pathway of bioactivation. The cy-

totoxicity and carcinogenicity of many chemicals is associated with the formation of electrophiles and the ensuing alkylation or acylation of tissue constituents such as protein, lipid, or DNA. Reactive intermediates include such chemically diverse functionalities as epoxides, quinones, acyl halides, carbocations, and nitrenium ions. The metabolic formation of electrophiles may be catalyzed by many different enzymes, although the majority of cases elucidated to date involve cytochrome P450-mediated oxidations.

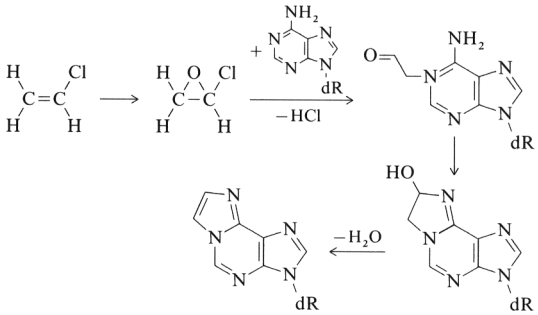


Figure 27. Bioactivation of vinyl chloride to chlorooxirane and reaction of the epoxide with critical macromolecules in the cell
dR = Deoxyribose

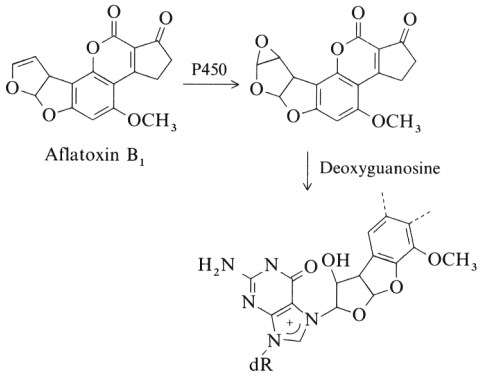


Figure 28. Bioactivation of aflatoxin B_1 to an electrophilic oxirane which results in the formation of DNA adducts and is believed to initiate tumor induction by aflatoxin B_1 in the liver

Cytochrome P450 catalyzes the transformation of olefins to reactive and electrophilic oxiranes. For example, the carcinogenicity of the industrial intermediate vinyl chloride (Fig. 27) and the fungal toxin vaflatoxin B1 (Fig. 28) are dependent on their transformation to electrophilic oxiranes [75, 76].

Carbocations are formed during the cytochrome P450-mediated oxidation of dialkyl nitrosamines. For example, the mutagen and potent carcinogen dimethylnitrosamine is hydroxylated by cytochrome P450 followed by loss of formaldehyde. Monomethylnitrosamine thus formed is unstable and rearranges to release an electrophilic carbocation (Fig. 29).

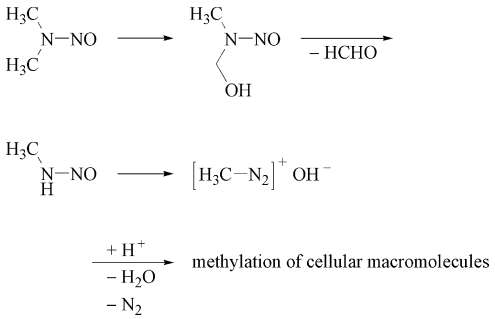


Figure 29. Bioactivation of dimethylnitrosamine to a methylating agent by cytochrome P450

Acyl halides are formed by the oxidation of carbon atoms bearing at least two halogen atoms. The initially formed products are unstable α-halohydrins, which lose hydrogen chloride and thus give reactive acyl halides. An example for the formation of acyl halides as reactive intermediates is the cytochrome P450-mediated oxidation of chloroform to phosgene [77] (Fig. 30).

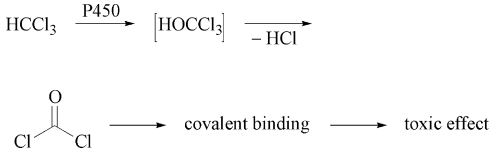


Figure 30. Bioactivation of chloroform by cytochrome P 450 mediated hydroxylation of a C–H bond

However, besides cytochromes P450, other monooxygenases such as flavin-dependent monooxygenase and of phase-II biotransformation enzymes such as UDP-glucuronyl transferases, sulfotransferases, or even the glutathione *S*-transferases may catalyze the bioactivation of xenobiotics [78]. For example, *N*-acetylamidofluorene is oxidized to *N*-hydroxyacetylamidofluorene by cytochrome P450. However, this metabolite is not electrophilic and requires further biotransformation via sul-

fate conjugation to the highly reactive *O*-sulfate ester. This sulfate ester fragments to a reactive intermediate (a nitrenium ion) which covalently binds to tissue constituents such as DNA (Fig. 31).

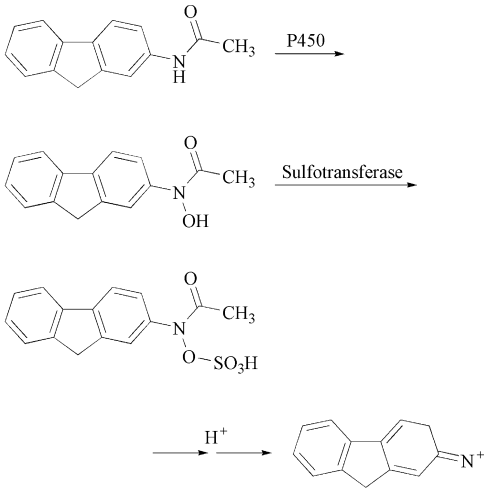


Figure 31. Bioactivation of acetylamidofluorene by cytochrome P450 and by UDP glucuronyl transferases. The glucuronide formed is acid-labile and decomposes to a nitrenium ion.

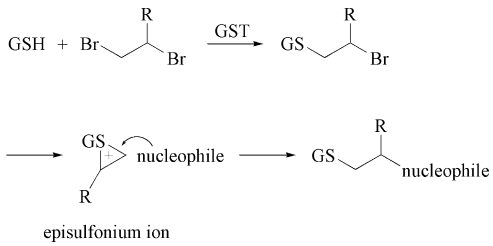


Figure 32. Bioactivation of 1,2-dibromoethane by glutathione conjugation to a reactive and electrophilic episulfonium ion

Some glutathione *S*-conjugates which are biosynthesized to detoxify electrophiles are toxic and mutagenic [79, 80]. 1,2-Dibromoethane is metabolized by glutathione conjugation to *S*-(2-bromoethyl)glutathione. Intramolecular displacement of the bromine on the adjacent carbon atom gives a highly strained, electrophilic episulfonium ion (Fig. 32).

Other toxic glutathione *S*-conjugates require processing by the enzymes of mercapturic acid formation to give electrophiles. A minor pathway in perchloroethene biotransformation results in *S*-(1,2,2-trichlorovinyl)glutathione [81]. This glutathione *S*-conjugate is cleaved by γ-glutamyl transpeptidase and dipeptidases to *S*-(1,2,2-trichlorovinyl)-L-cysteine, which is a substrate for renal cysteine conjugate β-lyase and transformed to pyruvate, ammonia, and a reactive thioketene, binding of which to cellular macromolecules is likely responsible for the renal toxicity of perchloroethene (Fig. 33).

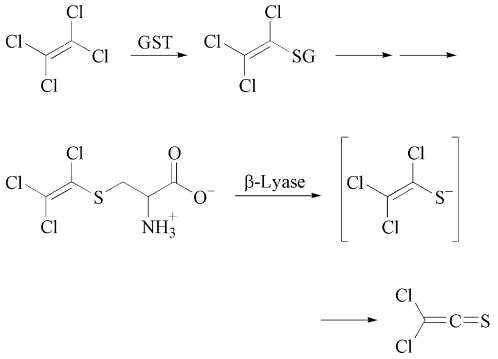


Figure 33. Bioactivation of perchloroethene by glutathione conjugation. The conjugate *S*-(1,2,2-trichlorovinyl)glutathione is biosynthesized in the liver, translocated to the kidney to be processed by γ-glutamyl transpeptidases and dipeptidases, and finally cleaved by cysteine-conjugate β-lyase to give dichlorothioketene.

Due to high concentrations of *S*-(1,2,2-trichlorovinyl)-L-cysteine obtained by active transport to the kidney, covalent binding of the dichlorothioketene formed via this pathway occurs only in the kidney; despite the presence of cysteine conjugate β-lyase in many other organs.

2.5.6.3. Biotransformation of Xenobiotics to Radicals

Free radicals are chemical species that may be formed by a one-electron oxidation to give a radical cation, by a one-electron reduction to give a radical anion, or by homolytic fission of a σ-bond to give a neutral radical.

Free radicals are highly reactive and, when formed in biological systems, are expected to react with a variety of tissue molecules. Radicals may abstract hydrogen atoms, undergo oxidation-reduction reactions, dimerizations and disproportionation reactions. Radicals may also participate in a chain mechanism, which is initiated by a reaction causing a free radical and propagated by a subsequence of reactions causing further radicals as products.

The toxic and tumorigenic solvent carbon tetrachloride is the outstanding example of a bioactivation reaction to a free radical. Carbon tetrachloride is biotransformed by a one-electron reduction to yield the trichloromethyl radical and chloride:

$$Cl_4C + e^- \rightarrow Cl_3C^{\bullet} + Cl^-$$

The trichloromethyl radical may abstract hydrogen atoms from tissue macromolecules to give chloroform, a proven metabolite of carbon tetrachloride, or may dimerize to give hexachloroethane, which is also a metabolite of carbon tetrachloride. Toxic effects of radicals formed during biotransformation reactions are lipid peroxidation and oxidative modification of proteins (see Section 2.5.6.4). Formation of radicals has been implicated in the bioactivation of many xenobiotics. Radicals may be formed by NADPH-dependent cytochrome P450 reductase, nitroreductases, or one-electron oxidations catalyzed by peroxidases such as prostaglanding synthetases. Formation of free radicals from tissue constituents also plays an important role in the toxic effects of ionizing radiation [82–85].

2.5.6.4. Formation of Reactive Oxygen Metabolites by Xenobiotics

Xenobiotic-induced formation of reduced oxygen metabolites such as the superoxide radical anion, hydrogen peroxide, and the hydroxyl radical has been implicated as a mechanism of producing cell damage, so-called oxidative stress [86–89]. The biotransformation of certain xenobiotics that are involved in redox cycles or undergo enzyme-catalyzed oxidation/reduction reactions may be associated with the production of reduced oxygen metabolites. 2-Methylnaphthoquinone (Menadione) has been intensively used to study the formation and cellular reactions of reduced oxygen metabolites. Menadione and other quinones undergo enzymatic redox cycling; these one-electron oxidation reactions are associated with the formation of the superoxide radical anion ($O^{\cdot 2-}$) by one electron reduction of triplet oxygen. In aqueous solution, superoxide is not particularly reactive, but dismutation or further reduction of superoxide may give rise to hydrogen peroxide (Fig. 34).

Hydrogen peroxide is also a poor oxidant in biological systems, but sufficiently stable to cross biological membranes. The toxicity of hydrogen peroxide is attributed to the formation of the hydroxyl radical by the Fenton reaction, catalyzed by metal ions such as Fe^{2+} (M = transition metal):

$$M^n + H_2O_2 \rightarrow M^{(n+1)} + HO^{\bullet} + HO^-$$

The highly reactive hydroxyl radical may then initiate cellular damage by radical-based mechanisms. Besides menadione, oxidative stress may also be initiated by other xenobiotics such as the bis-pyridinium herbicide paraquat and nitroheterocycles. Moreover, the formation of reduced oxygen metabolites plays an important role in host defense against infectious agents and in the initiation and propagation of certain diseases such as arteriosclerosis and polyarthritis.

Since oxygen radicals are also formed in low concentrations during cellular respiration, efficient mechanisms for their detoxication exist (see Section 2.5.6.5). Oxidative stress is thus only observed when the eqilibrium between oxidants and reductants is disturbed and detoxication mechanisms are overwhelmed.

2.5.6.5. Detoxication and Interactions of Reactive Metabolites with Cellular Macromolecules

Reactive intermediates formed inside cells may react with low and high molecular mass cellular constituents. These interactions may result in formation of less reactive chemicals and thus in detoxication, or may perturb important cellular functions and thus result in acute and/or chronic toxic effects such as necrosis or cancer. Usually, the interaction with low molecular mass constituents in the cell results in detoxication, whereas the irreversible interaction with cellular macromolecules results in adverse effects [70, 90 – 92].

Detoxication of reactive intermediates may be due to hydrolysis, glutathione conjugation, or interactions with cellular antioxidants. The reaction of electrophilic xenobiotics with the nucleophile water, present in high concentrations

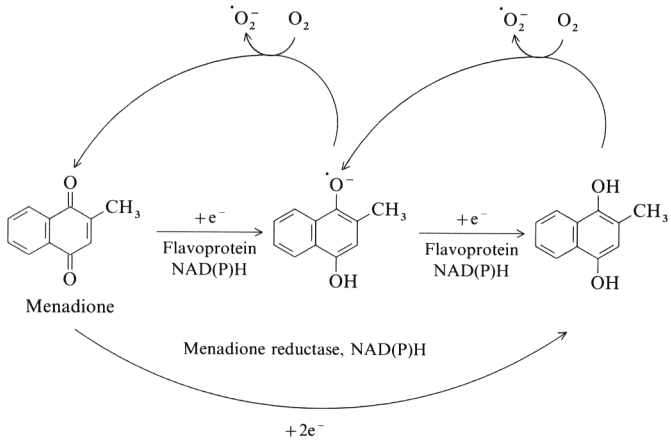


Figure 34. Biotransformation of menadione and induction of oxidative stress by reduction of triplet oxygen to the superoxide radical anion

in all cells, is the simplest form of detoxication. Many of the products thus formed are of low reactivity and may be rapidly excreted. For example, acyl halides formed by the oxidation of olefins such as perchloroethylene are hydrolyzed rapidly to halogenated carboxylic acids; only minor amounts of the intermediate acyl halide reacts with protein and lipids (Fig. 35).

Glutathione-dependent detoxication is an important mechanism for metabolically formed electrophiles, free radicals, and reduced oxygen metabolites [93–96]. Electrophiles react with the nucleophilic sulfur atom of glutathione in a spontaneous or enzyme-catalyzed reaction. Spontaneous reactions are only observed at appreciable rates with soft electrophiles (glutathione is a soft nucleophile); the conjugation of hard electrophiles with glutathione requires enzymatic catalysis; usually, the rates of conjugation catalyzed by glutathione *S*-transferase differ between hard and soft electrophiles; soft electrophiles are conjugated more efficiently. For example, the hard electrophile aflatoxin B_1 8,9-oxide does not spontaneously react with glutathione; only in the presence of a certain glutathione *S*-transferase enzyme is a glutathione *S*-conjugate of aflatoxin B_1 8,9-oxide formed. Species differences in the tumorigenesis of aflatoxin B_1 may serve to illustrate the important role of glutathione *S*-transferases in the expression of toxicity and carcinogenicity. Aflatoxin B_1 is a potent liver carcinogen in rats; in mice, aflatoxin B_1 is only weakly carcinogenic. The liver of mice contains a glutathione *S*-transferase which efficiently detoxifies aflatoxin B_1 8,9-oxide. This glutathione *S*-transferase enzyme is not present in rat liver; thus, the binding of aflatoxin B_1 8,9-oxide to rat liver DNA and liver carcinogenicity of aflatoxin B_1 are much higher in rats than in mice.

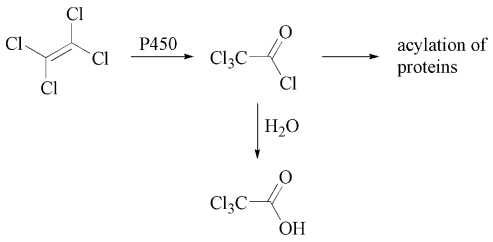


Figure 35. Biotransformation of tetrachloroethylene to trichloroacetyl chloride followed by hydrolysis to trichloroacetic acid, the major urinary metabolite formed from tetrachloroethylene. Only a small amount of the acyl halide formed reacts with proteins.

Glutathione also plays a major role in the detoxication of reactive oxygen metabolites and radicals. Selenium-dependent glutathione peroxidases are important enzymes catalyzing the detoxication of hydrogen peroxide. In the glutathione peroxidase catalyzed reaction, two moles of glutathione are oxidized to glutathione disulfide:

$$H_2O_2 + 2\,GSH \underset{\text{Glutathione reductase}}{\overset{\text{Glutathione peroxidase}}{\rightleftharpoons}} GSSG + H_2O$$

Glutathione can be recycled by the reduction of glutathione disulfide by glutathione reductase.

The copper- and zinc-dependent cytosolic and - manganese-dependent mitochondrial superoxide dismutases detoxify superoxide radical anions. Hydrogen peroxide formed by dismutation of superoxide is converted to water and oxygen and thus detoxified by catalase:

$$2\,H_2O_2 \xrightarrow{\text{Catalase}} 2\,H_2O + \tfrac{1}{2}\,O_2$$

Several cellular antioxidants also play a role in the detoxication of radicals. α-Tocopherol is an important lipophilic antioxidant, whose presence in lipid membranes prevents damage to lipid constituents (e.g. unsaturated fatt acids) by radicals. The hydroxyl radical, the superoxide radical anion, and peroxy radicals react with α-tocopherol to yield water, hydrogen peroxide, and hydroperoxides, which may be detoxified further by catalase and glutathione peroxidase. α-Tocopherol is transformed during these reactions to give a stable radical of comparatively low reactivity. Ascorbic acid is an important antioxidant present in the cytoplasm of the cell and may also participate in the detoxication of radicals.

2.5.6.6. Interaction of Reactive Intermediates with Cellular Macromolecules

Although a substantial body of information is available on the biotransformation of xenobiotics to reactive metabolites and the chemical nature of those metabolites, considerably less is known about how reactive intermediates interact with cellular constituents and how those interactions cause cell injury and cell death. The reaction of toxic metabolites may result in the formation of covalent bonds between the molecule and a cellular target molecule, or they may alter the target molecule without formation of a covalent bond, usually by oxidation or reduction [97].

Electrophilic metabolites may react with different nucleophilic sites in cells. Nucleophilic sites in cellular macromolecules are thiol and amino groups in proteins, amino groups in lipids, and oxygen and nitrogen atoms in the purine and pyrimidine bases of DNA. The formation of a covalent bond may permanently alter the structure and/or activity of the modified macromolecule and thus result in a toxic response. The complexity of the reaction of electrophilic metabolites with the various nucleophilic sites in cells may be interpreted on the basis of the concept of hard and soft electrophiles and nucleophiles (hard and soft acids and bases). The donor atom of a soft nucleophile is of high polarizability and low electronegativity, and is easily oxidized; the donor atom of a hard nucleophile is of low polarizability and high electronegativity. Hard electrophiles carry a high positive charge and have a small size; soft electrophiles are of low positive charge and large size. Soft electrophiles react predominantly with soft nucleophiles, and hard electrophiles with hard nucleophiles [98]. Thus, hard electrophiles formed during a biotransformation reaction (e.g., carbocations formed from dialkylnitrosamines) predominantly react with hard nucleophiles such as the oyxgen and nitrogen atoms of DNA, In contrast, soft electrophiles such as α,β-unsaturated carbonyl compounds (e.g., acrolein, benzoquinone) react predominatly with soft tissue nucleophiles such as the sulfhydryl groups of cysteine in proteins (Table 16).

Covalent interactions of xenobiotics with proteins occur with several nucleophilic nitrogen atoms; both alkylation and acylation reactions of amino acids have been reported as consequences of formation of reactive intermediates in cells. Besides the sulfur atom of cysteine, nitrogen atoms in the amino acids lysine, histidine, and valine are frequent targets for electrophilic metabolites. Consequences of the modifications may be inactivation of enzymes important for cellular function, changes in the tertiary structure of proteins, or changes in gene expression. Alkylation of the sulfhydryl-dependent enzymes of mitochondrial respiration is thought to play an important role in the initiation of mitochondrial dysfunction and thus cell damage.

Some modified proteins may also serve as immunogens, and hypersensitivity reactions, formation of immune complexes, and delayed hypersensitivities may be the consequences of protein adduct formation. Indeed, many drug- and chemical-related hypersensitivity reactions observed in clinical medicine are based on the formation of covalent protein adducts and their recognition as “foreign” by the immune system [99].

Oxidative stress produces mixed disulfides of proteins with low molecular mass thiols such as glutathione and thus alters protein structure

Table 16. Metabolically formed electrophiles and their prime targets for covalent binding in cells

Soft		→		Hard
		Nucleophile		
SH of cysteine or glutathione	sulfur of methionine	primary or secondary nitrogen atoms in peptides (lysine, arginine, or histidine)	amino groups of purines and pyrimidines in RNA and DNA	oxygen in purines and pyrimidines in DNA and RNA
		Electrophile		
α,β-Unsaturated carbonyls, quinones	epoxides, alkyl sulfates, alkyl halides	nitrenium ions	benzylic carbocations	aliphatic and aromatic carbocations

and function. In addition, oxidants and radicals promote the oxidation of amino acids in proteins, which may increase the susceptibility of these proteins to proteolysis [100, 101]. Increased protein oxidation has been implicated in cellular aging and in the mechanisms of toxicity of several redox-active transition metals.

Radicals formed during the biotransformation of xenobiotics may abstract hydrogen atoms from cellular components [82]. The abstraction of hydrogen atoms from polyunsaturated fatty acids of lipids results in a process termed lipid peroxidation. The fatty acid radicals thus formed may react with molecular oxygen to give peroxy radicals and further to hydroperoxides. The initiated radical chain reactions cause the cleavage of carbon–carbon bonds in the fatty acids to short fragments such as α,β-unsaturated carbonyl compounds [102, 103] (see Section 3.3.1).

The disruption of membranes and the formation of toxic hydroperoxides and α,β-unsaturated carbonyl compounds may cause disruptions in cellular calcium homeostasis and thus cause biochemical changes that ultimately lead to cell death [104, 105]. The reaction of electrophilic metabolites with DNA constituents results in the formation of altered purine and pyrimidine bases or other DNA damage such as DNA strand breaks or loss of single bases from the double helix. Many of these modifications are "premutagenic lesions". After gene expression, these lesions may be translated into mutations [106]. Mutations in certain genes are considered to be the basis for the evolution of neoplastic cells and cancer and thus play a major role in chemical carcinogenesis. Other types of DNA damage may result in the activation of genes important for cellular differentiation or other regulatory functions. Electrophilic intermediates alkylate the nitrogen and oxygen atoms of the purine and pyrimidine bases in DNA; deoxyguanosine is often preferentially alkylated. The site of alkylation of a certain base in DNA is again dependent on the electrophilicity of

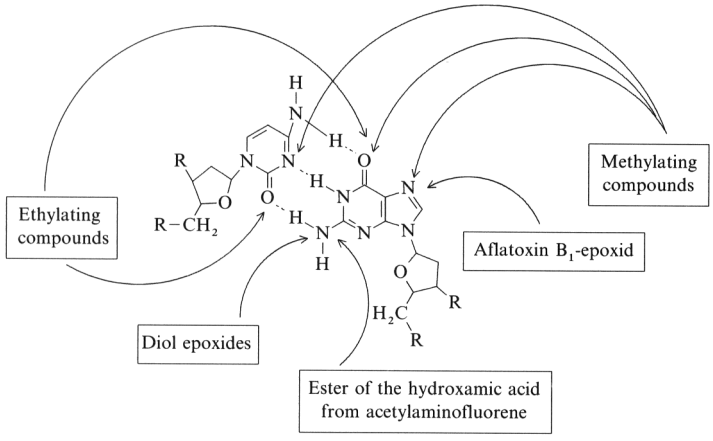


Figure 36. Regioselectivity of DNA alkylation by different electrophiles

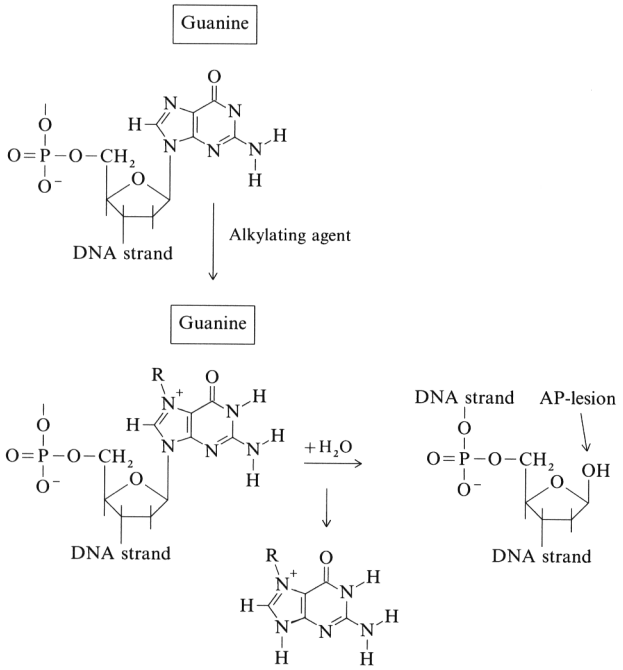
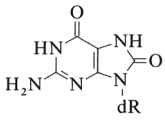


Figure 37. Labilization of the bond of guanosine to the DNA backbone by alkylation of the N-7 position resulting in the loss of the modified guanosine from DNA and the formation of an apurinic site (AP)

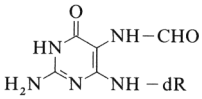
the alkylation agent; hard electrophiles preferentially react with the oxygen atoms of guanosine, while soft electrophiles alkylate the exocyclic amino groups (Fig. 36).

The pattern of base alkylation is different when DNA is modified in biological systems or when isolated DNA, nucleosides, and nucleotides are treated with the xenobiotic or its metabolite(s). Regioselectivity is further modified by solvents, buffer salts, and concentration of reactants. Certain modifications of deoxyguanosine result in the labilization of the glycosidic bond; loss of the deoxyguanosine derivate results in an "apurinic site" in DNA other chemical modifications may result in labilization of the five-membered ring and ring opening after reaction with water (Fig. 37).

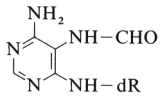
Radicals formed as reactive intermediates may also cause DNA damage. Besides DNA strand breaks, which have been frequently observed, the reaction of oxygen-derived radicals may also result in the oxidation of purine and pyrimidine nucleotides. Due to the development of sensitive techniques for the characterisation of oxidative modifications in DNA, a number of modified bases have been identified:



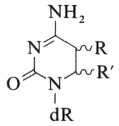
8-Hydroxydeoxyguanosine
(7,8-Dihydro-8-oxodeoxyguanosine)



Formamidopyrimidine-deoxyguanosine



Formamidopyrimidine-deoxyadenosine



R,R′ = H, OH, OOH
dR = Deoxyribose

5,6-Dihydrodeoxycytidine

8-Hydroxydeoxyguanosine, a premutagenic modification, is considered as one of the more important lesions induced by oxidative DNA damage; because sensitive methods are available

for its quantification, it can serve as a marker for the extent of oxidative DNA modification caused by a xenobiotic or by other processes. DNA oxidation has also been implicated in aging; an increase in oxidative DNA modifications may occur with age due the decreased availability of antioxidants in cells of aging mammals. Several theories suggest a correlation between increased oxidative DNA damage and the increased incidence of tumors in the aged population [107, 108].

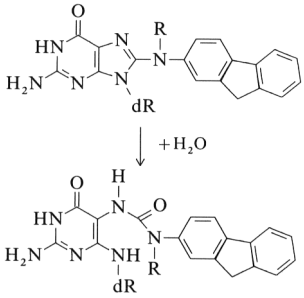


Figure 38. Ring opening of guanosine in DNA by alkylation of the N-7 position

2.5.7. Factors Modifying Biotransformation and Bioactivation

The biotransformation of xenobiotics may be modified by a variety of factors both intrinsic and extrinsic to the normal functioning of the organism. The changes in the extent of biotransformation may have profound effects on the toxicity of a specific chemical. When biotransformation results in detoxication and rapid excretion, increased rates of biotransformation will decrease toxicity. On the other hand, the toxicity of a chemical bioactivated to reactive intermediates will increase on enhancing biotransformation. A great variety of factors have been shown to influence the extent of biotransformation; many of the effects listed below have been primarily decribed in experimental animals. However, observations in humans (e.g,. after drug treatment) indicate that similar effects, albeit not of the same magnitude or duration, must occur in humans.

2.5.7.1. Host Factors Affecting Biotransformation

Enzyme Induction. The activity of biotransformation enzymes can be enhanced by pretreatment with a range of structurally different chemicals. These chemicals can be drugs, pesticides, natural products, environmental contaminants, and even ethanol. The enhanced enzyme activities and the increased enzyme concentrations may results from increased de novo synthesis of the protein, reduced degradation, or from other, often unknown effects. An increase in the concentration of an biotransformation enzyme in the organism, a certain organ or cell type is termed "enzyme induction" [109, 110]. Several hundred different chemicals have been demonstrated to increase the biotransformation of other xenobiotics and to act as enzyme inducers. The majority of these studies focused on the induction of microsomal monooxygenases, mainly cytochrome P450 enzymes; however, other membrane bound enzymes such as UDP-glucuronyl transferases may also be induced. Glutathione *S*-transferases are the only cytosolic biotransformation enzymes whose activities may be increased by the administration of inducers to experimental animals (Table 17).

Table 17. Inducers of the enzymes of biotransformation and enzymes whose cellular concentrations are increased by pretreatment

Inducing agent	Induced enzymes
2,3,7,8-Tetrachlorodibenzodioxin	cytochrome P450, UDPglucuronyltransferase
Ethanol	cytochrome P450
Phenobarbital	cytochrome P450, epoxide hydrolase, UDP-glucuronyltransferases
trans-Stilbenoxide	epoxide hydrolase
3-Methylcholanthrene	cytochrome P450, UDPglucuronyltransferases

The onset, magnitude, and duration of increases in the concentration of biotransformation enzymes after the administration of an inducer and the associated biochemical and morphological effects depend on the chemical nature of the inducing agent, dose, and time of administration. For example, the time required for maximum induction of specific cytochrome P450 enzymes by the classical inducers phenobarbital and 3-methylcholanthrene are different. Moreover, besides increases in the activity of hepatic monooxygenases, phenobarbital administration results in marked hepatic hypertrophy and proliferation of the smooth endoplasmatic reticulum; these effects are absent in animals

treated with 3-methylcholanthren. Induction by parenteral application of 3-methylcholanthren results in maximum enzyme concentrations in the liver within 48 h, whereas maximal induction by parenteral application of hypnotic doses of phenobarbital requires up to 5 d.

Enzyme induction is reversible after withdrawal of the inducing agent, and the enzyme activities return to basal levels over a characteristic time span. Again, this time span is dependent on the chemical nature of the inducing agent. For example, cessation of phenobarbital treatment will result in a decline of enzyme activities to basal levels within one to two weeks. The mechanisms of enzyme induction are complicated and only partially understood. Apparently, different chemicals influence the activities of the biotransformation enzymes by different mechanims; even the effect of a specific chemical on different enzymes may be due to separate mechanisms (Table 18) [111].

Table 18. Mechanisms of cytochrome P450 enzyme induction by different xenobiotics

Cytochrome P450	Inducing agent	Mechanism of induction
1A1	2,3,7,8-tetra-chlorodibenzodioxin	increased gene transcription
1A2	3-methylcholanthrene	stabilization of messenger RNA
2B1, 2B2	phenobarbital	increased gene transcription
2E1	ethanol, acetone	protein stabilization
3A1	dexamethasone	increased gene transcription, independent of glucocorticoid receptor
3A1	triacetyloleandomycin	protein stabilization
4A1	clofibrate	increased gene transcription, receptor mediated

Only a few mechanisms are well understood. Modulation of gene expression seems to be the basis for many inductive effects. For example, induction of cytochrome P450 1A1 is prevented by inhibitors of protein synthesis. Moreover, studies using the potent inducing agent 2,3,7,8-tetrachlorodibenzodioxin identified a high-affinity binding protein with the properties of a receptor for 2,3,7,8-tetrachlorodibenzodioxin in rat liver cytosol [112, 113]. Binding of 2,3,7,8-tetrachlorodibenzodioxin to this protein results, after further interaction with other proteins, in the translocation of the formed complex from the cytosol to the nucleus. This translocation is followed by interaction with specific recognition sites on the genome, transcription, and translation of the specific gene for cytochrome P450 1A1 and for other biotransformation enzymes such as UDP-glucuronyl transferases (Fig. 39).

In contrast, the mechanisms of enzyme induction by phenobarbital and, for example, ethanol are not defined. A specific receptor for phenobarbital could not be demonstrated, but some experiments suggest involvement of the glucocorticoid receptor in phenobarbital-mediated enzyme induction. Ethanol and other inducers seem to stabilize the cytochrome P450 2E1 protein against degradation by an unknown mechanism.

Enzyme Inhibition. The decrease in the activity of specific biotransformation enzymes is termed inhibition of biotransformation. As noted above, inhibition of biotransformation may increase or decrease the toxicity of a xenobiotic. For example, the inhibition of cytochrome P450 by 2-(diethylamino)ethyl-2,2-diphenylpentanoate (SKF-525A) causes an increase in hexobarbital sleeping time, but a decrease in the hepatoxicity of carbon tetrachloride.

Several mechanisms responsible for inhibition of xenobiotic metabolizing enzymes are operative [114, 115]. Besides inhibition of protein synthesis and thus synthesis of the enzyme, xenobiotics may irreversibly bind to the active site of the enzyme. This process is termed suicide inhibition [116]. Following activation of the xenobiotic by the enzyme, the reactive intermediate formed reacts with constituents of the enzyme at or near the active site, thus blocking further catalytic activity. This effect has been demonstrated with several xenobiotics which are inhibitors of cytochrome P450, such as alkenes and compounds containing allylic and acetylenic derivatives. For example, ethylene oxide, the reactive metabolite formed by cytochrome P450 catalyzed oxidation of ethylene, may alkylate the pyrrole nitrogen atoms in the heme moiety and thus result in heme destruction.

During exposures to mixtures, chemicals with high affinity to certain biotransformation enzymes will be preferentially metabolized, and thus the biotransformation of other constituents

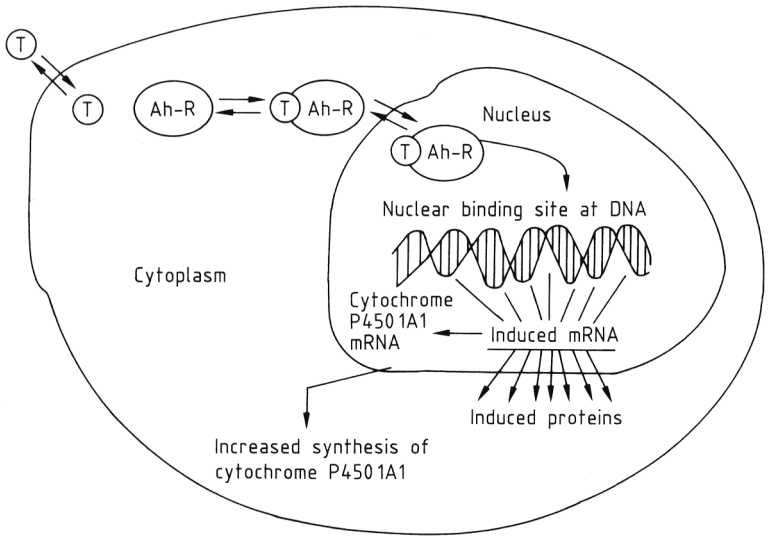


Figure 39. The Ah receptor (Ah-R) and mechanism of enzyme induction by 2,3,7,8-tetrachlorodibenzodioxin (T)

of the mixture will be reduced or even totally inhibited. This reduced biotransformation may also alter the toxicity of a chemical present in a mixture compared with that of the pure chemical. For example, coadministration of ethanol markedly reduces the toxic effects of methanol (metabolic acidosis, reversible or even permanent blindness). These toxic effects are caused by the oxidation of methanol to formic acid as toxic metabolite; formic acid accumulates in the organism and damages the optical nerve. Oxidation of methanol to formaldehyde is competitively blocked by administration of ethanol; under these circumstances, the toxic metabolite formic acid can not be formed; methanol is excreted from the organism unchanged in the urine and by exhalation.

Inhibition of some biotransformation enzymes may also be caused by effects on the tissue levels of necessary cofactors. The availability of glutathione for conjugation is reduced by blocking glutathione biosynthesis; diethyl maleate and some other chemicals deplete intracellular glutathione concentrations by reacting with glutathione to give a glutathione *S*-conjugate. Pretreatment of experimental animals with these chemicals followed by the application of a xenobiotic which requires glutathione for detoxication will result in an increased toxic response. Moreover, large doses of nontoxic chemicals metabolized by sulfotransferases may deplete the cofactor for sulfate conjugation and may thus alter the disposition and, probably the toxicity, of other xenobiotics that undergo sulfate conjugation.

Genetic Differences in the Expression of Xenobiotic Metabolizing Enzymes. The ability of different animal species to metabolize xenobiotics is related to evolutionary development and therefore to different genetic constitution; thus, major species differences in the extent and pathways of biotransformation exist. These variations may be divided into qualitative and quantitative differences. Qualitative differences involve metabolic pathways and are related to species defects or peculiar reactions of a species. For example, guinea pigs do not have the enzymatic capacity to catalyze the last step in mercapturic acid formation, the *N*-acetylation of cysteine *S*-conjugates, and therefore excrete cysteine *S*-conjugates as end products of this pathway. Certain species such as cats do not have the capability to form glucuronides from xenobiotics.

Quantitative variations are often due to species differences in gene and enzyme regulation. For example, interindividual differences have been decribed in humans in the biotransformation of many drugs. The *N*-acetylation of the tuberculostaticum Isoniazid has a genetic basis. Some individuals are homozygous for a reces-

sive gene, and this may result in the absence of isoniazid *N*-acetyltransferase, that is, they are "slow acetylators". In normal homozygotes or heterozygotes, "rapid acetylators", Isoniazid is rapidly transformed to the *N*-acetyl derivative. The *N*-acetyltransferase polymorphisms are correlated with different responses to Isoniazid-induced toxicities. This genetic polymorphism is also seen in human, 80% of the Japanese and Eskimos are "rapid acetylators"; whereas in some European populations only 40 to 60% are "rapid acetylators" [117]. Polymorphisms in the expression of cytochrome P450 1A1 and a specific glutathione *S*-transferase in human lung have been implicated in an increased rate of lung cancer in individuals expressing high cytochrome P450 1A1 and deficient in this glutathione *S*-tansferase. Cytochrome P450 1A1 bioactivates aromatic hydrocarbons present in cigarette smoke to yield electrophiles; glutathione *S*-transferase detoxifies these metabolites.

Influence of sex on biotransformation reactions. Sex differences in the extent and pathways of biotransformation may be based on sex-dependent expression of certain biotransformation enzymes. For example, adult male rats metabolize many xenobiotics at higher rates than females; both phase-I and phase-II biotransformation reactions seem to be influenced by sex-dependent factors. With cytochrome P450, at least three different hepatic enzymes have been demonstrated to be under the control of sex-hormones (Table 19).

Table 19. Sex hormone-dependent hepatic cytochrome P450 in the rat

Enzyme	Sex specificity	Remarks
P450 2D	female	expressed at a hormone-independent basal rate, stimulated by estrogen, suppressed by androgen
P450 2C	male	
P450 3A	male	neonatally imprinted by androgen

In addition to the liver, sex differences in biotransformation are also found in extrahepatic tissues such as the kidney and may be responsible for sex-specific toxic effects of xenobiotics in these organs. For example, the kidneys of male mice contain a cytochrome P450 enzyme which bioactivates chloroform, acetaminophen, and 1,1-dichloroethene to reactive intermediates. The enzyme is present only in much lower activity in female mice, which are thus not susceptible to the renal toxicity of these chemicals.

In experimental animals, sex differences in the expression of biotransformation enzymes usually become apparent at puberty and are maintained throughout adult life. Despite the relatively large sex-dependent variations seen in animal studies, sex seems not to have a profound influence on the biotransformation of chemicals in humans.

Dietary constituents and the biotransformation enzymes. Nutritional factors influencing biotransformation may be mineral deficiencies, vitamin deficiencies, protein content, starvation, and natural substances in the diet. Mineral deficiencies (calcium, copper, zinc) have been shown to reduce the activities of cytochromes P450. On the other hand, an excess of dietary iron has been observed to increase monooxygenase activity. Dietary cobalt, calcium, and manganese may increase the hepatic levels of glutathione and may thus influence glutathione *S*-conjugate formation. Several vitamins are directly or indirectly involved in the regulation of cytochrome P450. For example, diets deficient in vitamins C and E reduce the activity of monooxygenases, whereas deficiencies in other vitamins increase monooxygenase activity. Moreover, several vitamins serve as important cellular antioxidants and influence the energy and redox state of the cell and thus also affect biotransformation reactions.

Low-protein diets generally reduce the activity of cytochrome P450 and certain phase-II biotransformation reactions. Thus, the nutrient status may also modify the toxicity of xenobiotics. For example, dimethylnitrosamine is a potent hepatocarcinogen in rats kept on a high-protein diet; but almost without effect in rats kept on a low-protein diet. Food deprivation reduces the hepatic concentration of glutathione by as much as 50% due to reduction of glutathione biosynthesis. Thus, xenobiotics detoxified by glutathione conjugation are more toxic in starved than in fed animals. Fasting has also been shown to increase the levels of cytochrome P450 2E1, but decrease the levels of cytochrome P450

2C11. Several natural ingredients in the diet of laboratory animals such as indoles, diallyl disulfid,e and psoralens may increase the activities of cytochrome P450. However, some of these compounds may selectively increase some cytochrome P450 enzymes, but inhibit others. For example, diallyl disulfide, a constituent of garlic, has been shown to induce cytochrome P450 2B in rats, but inhibits cytochrome P450 2E1. Vegetable ingredients present in broccoli are potent inducers of phase-II biotransformation enzymes and thus increase the capacity of the organism to detoxify reactive intermediates. These natural ingredients are thought to play a major role in the anticarcinogenicity of diets rich in those vegetables.

2.5.7.2. Chemical-Related Factors that Influence Biotransformation

Xenobiotic-related factors influencing biotransformation are the physiocochemical properties (i.e., chemical structure including the presence of functional groups) and dose. The major determinant of the rate of biotransformation is the concentration of the substrate at the active site of the enzyme. This concentration is determined by structure and lipophilicity and by dose. Lipophilic xenobiotics readily cross cell membranes and are rapidly absorbed and distributed in the organism. Moreover, lipophilic xenobiotics show a higher partitioning into lipid membranes. These factors contribute to higher concentrations of lipophilic xenobiotics at the active center, especially of membrane-bound enzymes. The presence of functional groups also influences rates and routes of biotransformation. Certain functional groups may compete for the same substrate for conjugation; also, specific functional groups may undergo different reactions, as indicated in Figure 40 for *p*-aminobenzoic acid.

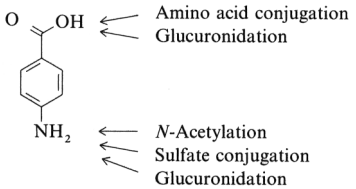


Figure 40. Possible biotransformation pathways for *p*-aminobenzoic acid

The presence of other specific functional groups may have a major effect on biotransformation and its regioselectivity. For example, the presence of trifluoromethyl groups in alkanes renders the adjacent methylene carbon atom almost inert to hydroxylation and strongly influences the regioselectivity of enzymatic hydroxylations on aromatic rings.

Dose is one of the most important factors determining rate and route of biotransformation for more complex molecules. Certain biotransformation enzymes have a high affinity but low capacity for a specific chemical, while others have a high capacity but low affinity. As dose increases, high-affinity, low-capacity enzymes will become staturated, and low-affinity, high capacity pathway(s) will biotransform a larger percentage of dose [118].

2.5.8. Elimination of Xenobiotics and their Metabolites

The evolution of complex forms of life necessitated the development of specialized mechanisms to eliminate waste products formed from endogenous compounds and to prevent the accumulation of toxic xenobiotics present taken up in the diet. Excretion of wastes by the earlier forms of aquatic life was largely passive and involved the loss of large volumes of water and nutrients. For land-living animals, conservation of water, minerals, and nutrients was necessary for survival. Therefore, complex mechanisms for the elimination of both endogenous chemicals and xenobiotics evolved. A wide variety of xenobiotics can be handled by evolved excretory mechanisms and can thus be efficiently eliminated from the body.

Xenobiotics may be excreted as the parent compound, as metabolites, and/or as conjugates formed in phase-II biotransformation reactions. A major route of excretion of xenobiotics is via the kidney, and in some cases the urinary elimination of parent compound or metabolite can be used to determine absorbed dose. The kidney is the only organ which functions almost exclusively as an organ of elimination. The cells of the liver have more varied functions than those found in the kidney; however, the liver also plays an important role in the excretion of chemicals not effectively eliminated by the kidneys. Chem-

icals may be eliminated from the liver into bile and thus be finally excreted with feces. Active transport mechanisms, that is, transport against a concentration gradient, play a major role in renal and hepatic excretion of xenobiotics. In contrast, in most other organs which may serve as excretory systems for xenobiotics, passive excretion mechanisms are operative. For example, volatile chemicals and metabolites may be eliminated in expired air; this route is quantitatively significant for some solvents and inhalation anesthetics. Specific xenobiotics may also be excreted in sweat, saliva, and milk.

2.5.8.1. Renal Excretion

The kidneys are the only organs that are primarily designed for excretion. The function of these organs accounts for the elimination of most of the byproducts of normal metabolism and most of the polar xenobiotics and metabolites of lipophilic xenobiotics to which humans and experimental animals are exposed [119]. The kidney is a complex structure which consist of a number of different cell types [119 – 121]. Essentially, the kidney filters the bood and all components present in blood with a molecular mass of less than 50 000 (depending on structure and charge) enter the tubular system; there, important nutrients and most of the filtered water are recovered. Only a small fraction of the primary filtrate is excreted as urine (one to two liters per day). The human kidney consists of approximately two million nephrons, which are the functional units that filter the blood and the recover essential nutrients. The structure and components of a single nephron are shown in Figure 41.

Glomerular Filtration. Renal excretion is the product of three complex and interactive processes: glomerular filtration, tubular reabsorption, and tubular secretion. Glomerular filtration is the passive filtering of the plasma as a result of its passage through glomerular pores (7–10 nm in diameter) under hydrostatic pressure generated by the heart. The average rate of glomerular filtration in adults is 125 ml/min or almost 200 liters/d. Glomerular filtration shows little specificity other than molecular size, and free solutes in the plasma that pass through the glomerular pores will all appear in the filtrate. Only protein-bound low molecular mass xenobiotics will not appear in the filtrate and remain in blood. Glomerular filtration is influenced by factors that affect the hydrostatic pressure or integrity of the glomerulus; thus, these factors may result in elevated plasma concentrations of excretory products formed from endogenous chemicals and from xenobiotics.

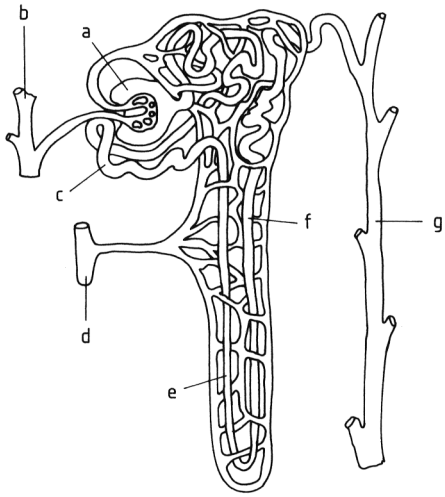


Figure 41. Structure of a nephron
a) Glomerulus; b) Renal artery; c) Proximal tubule; d) Renal vein; e) Loop of Henle; f) Distal tubule; g) Collecting duct

Tubular Reabsorption. The daily volume of glomerular filtrate exceeds that of the total body water by a factor of four and contains many necessary nutrients such as glucose, amino acids, and salt; therefore, most of the glomerular filtrate must be recovered. Thus, the second major process occuring in the kidney is tubular reabsorption. A number of discrete mechanisms, both active and passive and of varying degrees of specificity, are involved in tubular reabsorption. Many of these reabsorptive mechanisms are located in the cells of the proximal segments of the tubules. These cells account for the reabsorption of 65–90% of the gIomerular filtrate. Glucose, certain cations, low molecular mass proteins, amino acids, and organic acids are actively reabsorbed. Water and chloride are passively reabsorbed as a result of the osmotic and electrochemical gradients generated by the active transport of sodium and potassium. The osmolarity of the fluid in the collecting duct is

regulated in the loops of Henle; most of the remaining water and ions are reabsorbed in the distal tubules and collecting ducts. The rate of reabsorption in these segments of the proximal tubule is regulated to maintain the osmolar concentration of the blood. Most xenobiotics are also reabsorbed after glomerular filtration by passive diffusion during passage through the nephron. Passive tubular reabsorption of lipophilic xenobiotics is therefore greater than the reabsorption of polar xenobiotics or endogenous wastes.

Tubular Secretion. Xenobiotics present in blood may also be excreted by the kidney by tubular secretion. This secretion transports xenobiotics from the peritubular fluid (blood) to the lumen (urine) in the tubule. Tubular secretion is often selective; active transport mechanisms account for the secretion of many organic acids, including glucuronides and sulfates, and strong organic bases.

The secretion of weak bases and some weak acids may also occur by a passive mechanism that utilizes pH differences between peritubular fluid and urine. At the pH of the tubular lumen, these compounds become ionized and do not diffuse back across the cell wall.

Factors Affecting Renal Excretion of Xenobiotics. Xenobiotics are excreted by the same mechanisms which eliminate endogenous wastes; highly polar xenobiotics in plasma water are removed primarily by glomerular filtration and excreted in the urine with minimal involvement of tubular reabsorption or secretion. The rate of renal elimination of most of these xenobiotics is largely dependent on the rate of glomerular filtration. Since lipophilic compounds cross cell membranes more readily, they distribute into a much larger tissue volume than polar compounds, which are more likely to be restricted to the vascular volume. However, lipophilic xenobiotics metabolized to more polar compounds are usually rapidly returned to the circulation and are readily excreted. Therefore, the rate of metabolism of a xenobiotic may also play an important role in its rate of excretion.

2.5.8.2. Hepatic Excretion

Nutrients and xenobiotics are delivered from the gastrointestinal tract to the liver by the portal vein to be biotransformed there. Thus the liver is located between the intestinal tract and the general circulation and ideally located for the biotransformation of nutrients and xenobiotics taken up by this route. Besides participating in the biotransformation of xenobiotics, the liver is a major excretory organ and contributes to the excretion of many xenobiotics by eliminating them with bile into the gut and thus into feces [122–125].

The bulk of the liver consists of cells arranged in plates two cells thick. These plates are arranged radially around the terminal branches of the hepatic veins and are exposed to blood from the portal vein and hepatic artery flowing through interconnecting spaces referred to as hepatic sinusoids. The sinusoidal walls are freely permeable even to relatively large particles; special transport may only play a role in the uptake of certain anions from the blood. The epithelial hepatic cell is the smallest unit of the liver and accounts for most of the varied functions of this organ, including storage, secretion, biotransformation, and excretion (Fig. 42).

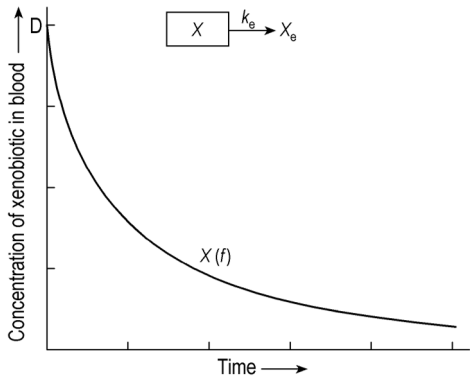


Figure 42. One-compartment model with first-order elimination and instantaneous absorption

Bile formation is thought to be the result of active transport of certain ionized compounds and passive transport of other solutes and water, which follow a concentration or electrochemical gradient. Active secretion of anions and cations appears to be controlled by different mechanisms, but the compounds actively excreted are usually amphipathic molecules and have both polar and nonpolar portions in their structures. Bile acids are the classical example of endogenous amphipathic molecules. Conjugates of lipophilic xenobiotics are also examples

of amphipathic molecules, and many of these conjugates are ionized, a fact that facilitates excretion from the hepatocyte by active transport mechanisms.

As a result of both active and passive secretion into the bile, xenobiotics excreted into bile may be classified into different groups. Solutes found in bile may be divided according to their concentration in bile versus blood. For example, the excretion of Na^+, K^+, Cl^-, and glucose, for which the bile/blood ratio is close to unity, is thought to be passive. Conjugates of xenobiotics and bile acids have a bile/blood ratio of greater than 10 and are thought to be actively excreted from liver into bile. Other compounds such as proteins, inulin, sucrose, and phosphates do not cross the canalicular membrane of hepatocytes and have a bile/blood ratio of much less than one. Bile secreted by the liver cells into the bile canaliculi flows into the narrowest branches of the bile duct, the cholangioles, then into the hepatic duct, which carries the bile to the gallbladder and finally into the intestine. Lipophilic xenobiotics may appear in bile at low concentrations prior to metabolism. However, a compound absorbed from the stomach or intestine after ingestion is likely reabsorbed from the intestine if it is secreted in bile prior to structural modifications by biotransformation. Moreover, conjugates excreted into bile, such as glucuronides, may be hydrolyzed by enzymes present in the bacteria of the intestinal microflora, and the aglycone may be reabsorbed. Most xenobiotics reabsorbed from the intestine are returned to the liver. The process of excretion into bile, reabsorption from the intestine, and return to the liver is termed enterohepatic circulation. Enterohepatic circulation serves as an efficient physiological recovery mechanism for bile acids and certain hormones. When xenobiotics are trapped in this cycle, their rate of excretion from the body may be significantly reduced and their toxicity may be significantly increased.

Effect of Molecular Mass on Hepatic Excretion of Xenobiotics. From the liver cell, xenobiotics may be excreted into bile or returned to blood. The molecular mass of nonvolatile organic xenobiotics or their metabolites determines the primary route by which they are excreted from the hepatocyte. In the rat, xenobiotics, their metabolites and conjugates formed in phase-II reactions with molecular masses greater than 500 are most often excreted into bile, while xenobiotics and their metabolites with molecular masses of less than 350 return from the hepatocyte to blood and are thus delivered to the general circulation and to the kidney for excretion. In humans, the critical molecular mass threshold for biliary excretion is approximately 500.

2.5.8.3. Xenobiotic Elimination by the Lungs

Any xenobiotic present in blood with sufficient volatility will pass from the blood across the alveolar membrane into the air space of the lung and may be exhaled. The rate of elimination from blood of a volatile xenobiotic is dependent on the solubility of the xenobiotic in blood, the rate of respiration, and the blood flow to the lung. Xenobiotics like diethyl ether, which are highly soluble in blood, are rapidly eliminated by exhalation; their elimination may also be efficiently increased by a forced increase in the rate of respiration (hyperventilation). In contrast, volatile xenobiotics with low solubility in blood are only slowly cleared from the lung and thus from the body by exhalation, and their rate of exhalation may not be markedly influenced by hyperventilation. The proportionality among xenobiotic volatility, blood solubility, and the concentration of a volatile xenobiotic in the blood is utilized to quantitate blood alcohol content and thus estimate sobriety by breath analysis.

2.6. Toxicokinetics

The toxic response to chemical exposure depends particularly on the magnitude, duration, frequency, and route of exposure. These determine the amount of material to which an organism is exposed (the exposure dose) and hence the amount of material which can be absorbed (the absorbed dose). The latter determines the amount of material available for distribution and toxic metabolite formation, and hence the likelihood of inducing a toxic effect. Absorption and metabolite accumulation are opposed by elimination. All these factors define the disposition of the xenobiotic. The modeling and mathematical description of the course of disposition

of a potentially toxic xenobiotic in the organism with time is termed toxicokinetics. Most of the methodologies and principles applied in toxicokinetics were first used to model the kinetics of chemicals applied as drugs (pharmacokinetics).

The goal of both toxicokinetics and pharmacokinetics is to quantitate the dynamic course of xenobiotic absorption, distribution, biotransformation, and elimination processes in living organisms with time. Both the whole process of disposition or individual steps such as elimination may be characterized.

2.6.1. Pharmacokinetic Models

A pharmacokinetic or toxicokinetic model is a functional representation that has the ability to describe the movement of a xenobiotic over time in a real biological system [126–128]. A common way to describe the kinetics of drugs is to represent the body as a number of interconnected compartments which may or may not have an anatomical or physiological reality. These compartments represent all tissues, organs, and fluids in the body that are kinetically indistinguishable from each other. A compartment might be represented by a cluster of cells within an organ, an organ, the blood, or the whole body taken together. More recently, so called physiologically based pharmacokinetic and toxicokinetic models have been developed. These models permit the modeling of the time course of the concentration of a chemical in a tissue on the basis of physiological considerations and may be particulary useful in interspecies extrapolations such as are necessary in risk assessment.

2.6.1.1. One-Compartment Model

This simplest toxicokinetic model depicts the body as a single homogeneous unit within which a xenobiotic is uniformly distributed at all times. The toxicokinetics of a xenobiotic may be analyzed by a one-compartment model if the determined plasma concentration after a single dose decreases exponentially (Fig. 43; the plot of the logarithm of the plasma concentration versus time yields a straight line). In this model, elimination of a chemical from the body occurs by first-order processes. A mathematical description of the first-order process is

$$\frac{\mathrm{d}X}{\mathrm{d}t} = -k_{\mathrm{e}}X$$

where X is the amount in the body at time t and k_{e} is the rate constant for first-order elimination. In compartmental analysis, k_{e} is often referred to as an apparent first-order rate constant, to emphasize that the underlying processes may in reality only approximate first-order kinetics. For example, the xenobiotic might actually be eliminated by active biliary secretion, for which zero-order saturation kinetics would be observed under suitable conditions.

If the xenobiotic is not present in the organism and a known amount X_0 is rapidly administered, the total amount of the xenobiotic initially in the body will be approximately X_0 = total dose. The amount of the xenobiotic present in the organism at a certain time after administration is then

$$X\,(t) = X_0 \times \mathrm{e}^{-kt}$$

The first-order rate constant of elimination can be determined from the slope of the plot of the logarithmic plasma concentration versus time and can be used to estimate the half-life of elimination for the xenobiotic:

$$t_{1/2} = \frac{0.693}{k_{\mathrm{e}}}$$

Within seven half-lives, a xenobiotic is almost completely eliminated (99.2%), although theoretically complete elimination will never be achieved. Important characteristics of the first-order elimination of a xenobiotic according to the one-compartment model are:

1) The half-life of the xenobiotic is independent of dose; the semilogarithmic plot of the plasma concentration of the xenobiotic versus time yields a straight line.
2) The concentration of the xenobiotic in plasma decreases by a constant fraction per time unit.

Xenobiotics responsible for toxic effects are usually not injected into blood and intake is thus not instantaneous compared to distribution and elimination and a lag period before maximal concentration in plasma is observed. If intake also approximates a first-order absorption process, then the rates of absorption and elimination

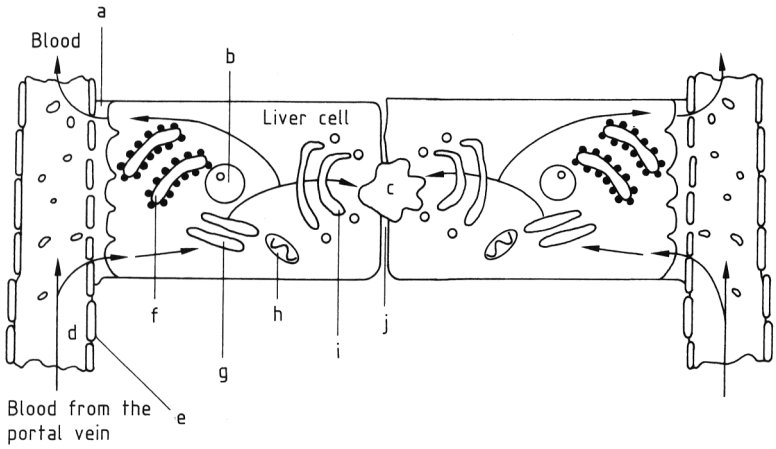


Figure 43. Structure of a liver cell (hepatocyte) and possible pathways for the uptake and elimination of xenobiotics and their metabolites
a) Space of Disse; b) Nucleus; c) Bile; d) Liver capillary; e) Sinusoidal wall; f) Rough endoplasmic reticulum; g) Smooth endoplasmic reticulum; h) Mitochondria; i) Golgy complex; j) Bile duct

will determine the time course of the plasma concentration (Fig. 44).

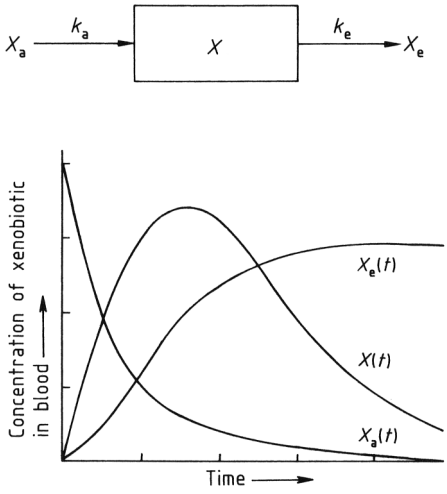


Figure 44. One-compartment model with first-order absorption and first-order elimination

The rate of change in the concentration of a xenobiotic in a one-compartment model with first-order absorption and elimination may be described by:

$$\frac{dX}{dt} = -k_e X_e + k_a X_a$$

where k_a is the rate constant of absorption, X_a is the amount of the xenobiotic present at the site of absorption, and k_e is the rate constant of elimination.

The rate constant of elimination can be experimentally determined by treating the elimination phase as if it occurred after the intravenous injection described above by extrapolation of the terminal straight line of the semilogarithmic plot to the ordinate at a putative X_0. By determing the plasma concentration of the xenobiotic at time points shortly after administration, the rate constant for absorption can be determined by plotting the difference between the early plasma concentrations and the extrapolated portion of the elimination curve.

2.6.1.2. Two-Compartment Model

lf a xenobiotic does not distribute and equilibrate throughout the body rapidly, a two-compartment model provides a better description of the kinetics of disposition. In this case, the semilogarithmic plot of plasma concentration versus time does not yield a straight line. In two-compartment models, the main or central compartment is assumed to represent the blood and highly perfused organs and tissues such as the liver, the heart, and the kidneys, which are in rapid distribution equilibrium with the blood, while the second or peripheral compartment corresponds to poorly perfused tissues such as muscles and fat. This model is depicted in Figure 45 and is called a two-compartment open pharmacokinetic model.

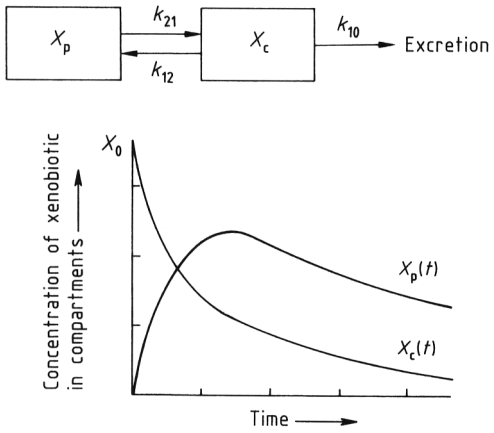


Figure 45. Two-compartment model for the distribution of a xenobiotic
X_c = concentration in the central compartment; X_p = concentration in the peripheral compartment; X_0 = initial dose; k_{21}, k_{12}, k_{10} = rate constants

The time course of the plasma concentration of the xenobiotic can be decribed by two overlying monoexponential terms of the type:

$$X = Ae^{-kt} + Be^{-k't}$$

where A and B are proportionality constants and k and k' are rate constants. Thus, the pharmacokinetics of a two-compartment model describe a biexponential decay in the amount of xenobiotic in the body. Analogous to a one-compartment model, a distribution equilibrium is assumed to exist within each compartment that can be expressed in terms of blood concentration. According to this equation, a plot of the logarithm of blood concentration versus time will yield a biphasic decay from which the constants A, B, k, and k' can be estimated either graphically or by nonlinear regression analysis. Once these experimental constants have been determined, the pharmacokinetic parameters can be calculated. The numerical values of these parameters can aid in assessing the relative importance of tissue distribution and elimination to the disposition of the xenobiotic.

2.6.2. Physiologically Based Pharmacokinetic Models

Although compartmental analysis models are convenient to use and provide useful descriptions of the overall time course of xenobiotic disposition, the practical limitations of curve fitting to experimental data generally restrict such models to one or two compartments. Alternatively, it is often possible to develop a compartmental model from physiological principles and thereby circumvent the inherent limitations of curve-fitting analysis. A physiological pharmacokinetic or toxicokinetic model is a mathematical description of the disposition of a xenobiotic in the organisms or in a part thereof (e.g., a specific organ) Such a model is constructed by using physiological and biochemical parameters such as blood flow rates, tissue and organ sizes, binding, and biotransformation rates [129–131]. These are generally more complex and require the specification of many parameters; thus, physiological compartmental models are still simplified representations of biological systems. In addition, the accurate determination of physical and biochemical parameters is often both difficult and inaccurate. However, the physiological framework provides several advantages: the physical definition of compartments and transfer rates facilitates the incorporation of existing knowledge about the quantitative behavior of biological systems into the model; physiological changes with time during chronic exposure to a xenobiotic, such as those due to physical growth, or induction of biotransformation and changes in excretion rates, can be introduced (Fig. 46).

Most important, however, a reasonable basis exists for extrapolating the kinetics of a xenobiotic to predict the disposition of a xenobiotic following various types and patterns of exposure, and to extrapolate from experimental data obtained in one species of experimental animals to other animal species and humans.

Physiology-based models use the concepts of mass balance and flow-limited transport under the following definitions: Blood serves to distribute a xenobiotic from the site of absorption to the other parts of the body. In the normal sequence of events, a chemical entering the bloodstream is often rapidly distributed within the blood, and its blood concentration can be considered to be essentially uniform.

The chemical enters and leaves the compartment with the blood flow and diffuses or is transported from blood to tissue and back. The chemical may also undergo a variety of physical interactions, such as binding to macromolecules, and the result is a partitioning between tissue and

blood that depends on the affinity of the chemical for each medium. Diffusion or transport directly between adjacent compartments, enzymatic biotransformations, and excretion may also occur. The net result of all of these changes is expressed by a mass-balance differential equation, which is simply a mathematical statement of the conservation of mass. Frequently, these mass balance equations can be greatly simplified, since many of the terms required may not apply to a particular compartment.

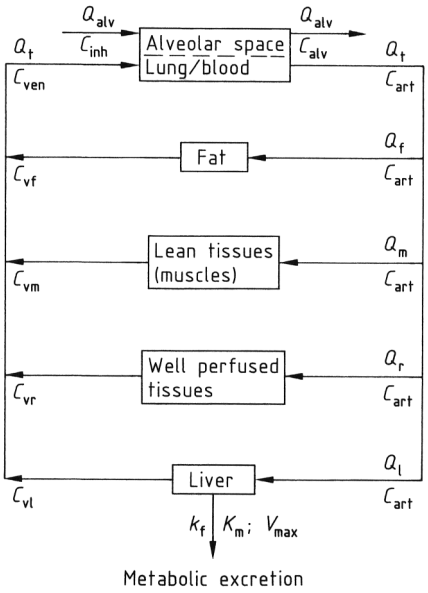


Figure 46. Schematic representation of a physiologically based toxicokinetic model

3. Mechanisms of Acute and Chronic Toxicity and Mechanisms of Chemical Carcinogenesis

3.1. Biochemical Basis of Toxicology

Since the 1960s, toxicology has moved from observing and classifying the harmful effects of chemicals in animals with the tools of pathology (descriptive toxicology), to a discipline able to explain the mechanisms of the basic changes in cell function responsible for toxic effects (mechanistic toxicology). This progress resulted from the widespread application of techniques and concepts from a range of basic sciences, most notably biochemistry and cell biology. Mechanistic explanations of toxic phenomena aid in the prevention of chemical or biological toxicity and provide a rational basis for the use of animal data to assess the anticipated consequences of human exposure to a particular chemical.

Many toxic compounds are chemically stable and produce their characteristic effects by interference with biochemical or physiological homeostatic mechanisms. Many adverse events are the consequence of disturbance of normal physiology and do not result in cell death. A xenobiotic may, for example, activate plasma membrane receptors and induce physiological signal transduction pathways. Induction of a normal cascade at the wrong time or to the wrong extent (too much) may cause undesirable or harmful effects. Toxic compounds interfering with homeostatic mechanisms do not necessarily cause cell death; however, the induced changes may have a harmful impact both on the altered cells and on the involved tissue or on the entire organ. Therefore, it is critical to have a proper knowledge on biochemical and molecular sites of action of the xenobiotics.

Cytotoxicity resulting in cell death is often the consequence of exposure to a harmful xenobiotic, but the number of cells which must be killed before the function of a tissue or organism is noticeably impaired is highly variable. Some cell types like the epithelia of the kidney and the liver have the ability to regenerate in response to damage, while others like neurons can not. Furthermore, some organs, such as the liver, lung, and kidney, have a substantial functional reserve capacity in excess of normal requirements, and normal function can be maintained even in the presence of extended necrosis.

In addition to cell death, disturbances in the regulation of cell division induced by toxic xenobiotics may have harmful long-term consequences for the organism affected. Nonlethal alterations in the genome of somatic cells can result in mutations and can lead to malignant transformation and tumor formation [132]. More recently, it has become clear that compounds not directly interacting with the genomic DNA can also produce cancer by so-called epigenetic mechanisms [133]. These may involve a proliferative response of epithelial cells to cytotoxicity, as is suggested to occur with high-dose carcinogens such as allyl isothiocyanate and chloroform, or a more direct action that enhances the rate of cell division in the absence

of cytotoxicity, as is seen with 2,3,7,8-tetrachlorodibenzodioxin. Increased cell replication, whatever the cause, is accompanied by increased chance of unrepaired DNA lesions that may be fixed as mutations. Hyperplasia has long been suspected of preceding neoplasia, but the inevitability of such a progression has never been established on a pathological basis alone. Even in absence of foreign compounds, DNA is damaged to a considerable extent by reactive oxygen species formed during different biochemical processes in the cell, it may thus be speculated that nongenotoxic carcinogens act by enhancing the likelihood of this normal DNA damage being fixed as a mutation and leading to cancer [134].

Having established the fundamental differences between toxic chemicals which act through physiological inbalance, through cytotoxicity, or by causing alterations in cell proliferation, we now consider some sites or chemical reaction mechanisms of toxic action. The following major mechanisms are neither comprehensive nor mutually exclusive.

3.2. Receptor-Ligand Interactions

3.2.1. Basic Interactions

Some xenobiotics can interact with physiological receptors due to structural similarities to endogenous compounds. The toxic effects of these chemicals are related to their ability to interfere with normal receptor–ligand interactions either as agonists or as antagonists of the physiological ligand. Receptors are macromolecular components, most often proteins, of tissues which interact with specific endogenous ligands or structurally related xenobiotics to induce a cascade of biochemical events and produce characteristic biological effects. The binding between a receptor (R) and a ligand (L) is usually reversible and can be described by the equilibrium reaction:

$$\mathrm{R} + \mathrm{L} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{RL}$$

The dissociation constant K_d that describes this relationship is thus given by:

$$K_\mathrm{d} = \frac{k_1}{k_2} = \frac{[\mathrm{L}]\,[\mathrm{R}]}{[\mathrm{LR}]}$$

where [L], [R], and [LR] are the concentrations of ligand, unbound receptor, and ligand-bound receptor, respectively. The affinity of the ligand to a certain receptor is proportional to $1/K_\mathrm{d}$. The ligand may be an endogenous substance that interacts with the receptor to produce a normal physiological response, or it may be a xenobiotic that may either elicit (agonist) or block (antagonist) the response.

Receptor–ligand interactions are generally highly stereospecific. Usually, small changes in the chemical structure of the ligand can Drastically influence its capability to bind to the receptor and thus reduce or completely abolish the effect elicited by the ligand–receptor interaction. In other cases, changes in the chemical structure may have an important impact on the response without altering the binding of the xenobiotic to the relevant receptor. Differences in the extent of activity are not only observed with structurally distinct chemicals but also with chemicals that are chiral and thus may be present as racemic mixtures of stereoisomers. Synthetic chemicals with chiral centers generally contain both enantiomers, often in a 1:1 ratio, yet in many instances only one of the enantiomers is biologically active. In pharmacological or toxicological studies, the inactive or weakly active enantiomer should be viewed as an "impurity" which may confound the interpretation of results obtained with the racemic mixture. Because the enzymes responsible for biotransformation of xenobiotics also contain active sites with specific steric requirements, stereoisomers may be differentially biotransformed. The selective biotransformation of only one enantiomer may markedly alter the potency and efficacy of one enantiomer compared with another. Stereoselective differences in action among enantiomers should not be surprising, if one considers that these chemicals are mirror images, much as the right hand is of the left, and that receptors have a physical orientation that can be compared with a glove. Although the left-hand and right-hand gloves look remarkably similar, they do not fit both hands equally well. However, these theoretical considerations do not imply that stereoisomers always elicit different responses. Since the chiral center of a stereoisomer is not necessarily involved with the active site of the receptor, chirality does not always results in differences in toxic response among enantiomers.

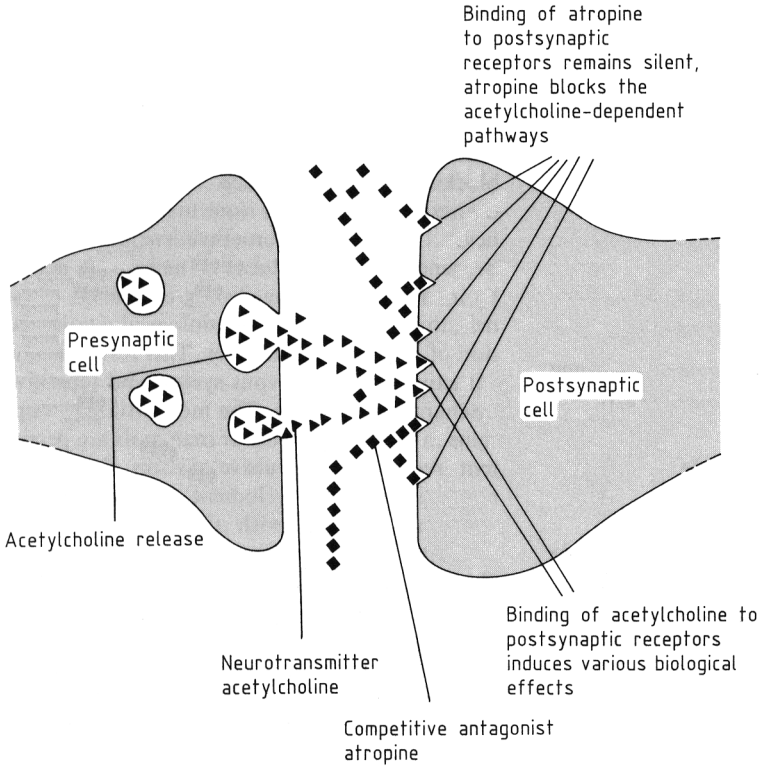


Figure 47. Induction of physiological response(s) by binding of the agonist acetylcholine to postsynaptic receptors; in contrast, binding of the antagonist atropine to the same receptors does not induce biological effects

The acute toxic effects of many xenobiotics are related directly to their ability to interfere with normal receptor–ligand interactions. This is most clearly the case with neurotoxins, acting within and outside the central nervous system (CNS). The proper function of the nervous system is highly dependent on a diverse array of receptor–ligand interactions. For example, the belladonna alkaloids atropine and scopolamine bind to and block the cholinergic receptors, the binding site of the physiological agonist acetylcholine (Fig. 47). The interactions of acetylcholine and atropine are an excellent example demonstrating that interference of a xenobiotic with physiological regulatory mechanisms may result both in desired (pharmacological) and an undesired (toxic) effects.

Since atropine itself can not elicit the physiological responses mediated by acetylcholine binding to this receptor, toxic effects may result. In clinical therapy, atropine is often used as an antispasmodic to reduce the hypermotile state of the gastrointestinal tract or the urinary bladder. Its anticholinergic effects are also utilized in cardiovascular pharmacology to decrease pathologically elevated heart rates. At the same time, however, blockage of acetylcholine-mediated physiological cascades may cause dry mouth, blurred vision, and constipation. In addition, in the CNS toxic effects of atropine include restlessness, confusion, hallucinations, and delirium. Blockage of the receptors for the neutral amino acid glycine by strychnine represents a further example of the important and, in this particular case, life-threatening agonist–antagonist interactions. Binding of glycine to its receptor induces an increased permeability of the plasma membrane to chloride ions resulting in hyperpolarization and reduced activity of nerve cells, that is, glycine exerts an inhibitory effect on the nervous system. Blockage of the glycine receptors by the antagonist strychnine, that does not elicit a response, results in hyperactivity and severe convulsions.

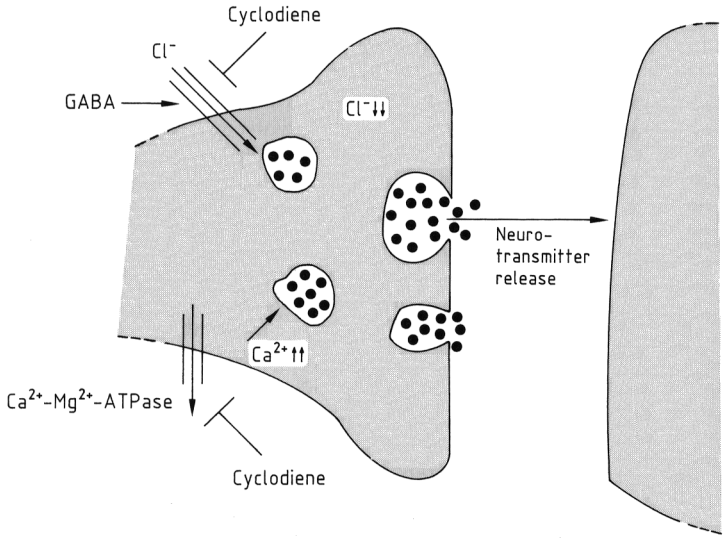


Figure 48. Sites of action of chlorinated cyclodiene insecticides on the plasma membrane of nerve cells

Table 20. Chemicals causing toxic response by interaction with receptors (Ah = aryl hydrocarbon)

Type of receptor	Chemical
Muscarinic receptor	atropine
Glycine receptor	strychnine
Ah receptor	TCDD
Peroxisome proliferator receptor	peroxisome proliferators (clofibrate, diethylhexylphthalate)

In addition to acute toxicity, chronic influences exerted by xenobiotics on steroid hormone homeostasis may result in adaptive and proliferative responses in certain tissues. The long-term consequences may involve impairment or loss of the physiological function. Moreover, binding to a specific receptor may cause changes in gene expression and contribute to tumor formation. For example, binding to the Ah receptor (the aryl-hydrocarbon receptor, named on the basis of its interaction with planar, polycyclic aromatic compounds) mediates many of the effects observed with the highly toxic and carcinogenic 2,3,7,8-tetrachlorodibenzodioxin (TCDD). A cytosolic binding protein (cytosolic receptor) has also been identified for peroxisome proliferators, such as clofibrate and diethylhexyl phthalate [135, 136]. This protein has DNA-binding domains and appears to activate gene transcription in the nucleus in a manner analogous to the steroid hormones. The increased cell proliferation caused in target organs such as the liver may be responsible for the carcinogenicity of many peroxisome proliferators. Characteristic examples of receptor–xenobiotic interactions with harmful acute or chronic consequences are summarized in Table 20.

3.2.2. Interference with Excitable Membrane Functions

The maintenance and stability of excitable membranes is essential to normal physiology. Excitable membranes are critical to the function of nerves and muscles due to their ability to generate and propagate action potentials. Action potentials are elicited by the exchange of ions between the intra- and extracellular compartments, and hence they depend on the normal activity of ion channels and membrane ion pumps.

Xenobiotics may perturb excitable membrane functions in many different ways. Xenobiotics can block sodium channels resulting in toxic effects such as paralysis. For example, tetrodotoxin from the the puffer fish irreversibly blocks the sodium channel along the nerve axon and thus prevents the inward sodium current of the action potential while leaving the outward potassium current unaffected. The marine toxin saxitoxin, which is

structurally quite different from tetrodotoxin, also produces its paralyzing effects by blocking sodium channels in excitable membranes in essentially the same manner. The insecticide dichlorodiphenyltrichloroethane (DDT) produces its neurotoxic action by interfering with the closing of sodium channels, which impairs the physiological repolarization of excitable membranes. This results in hyperactivity of the nervous system and repetitive discharges of neurons. The most striking symptoms in poisoned insects or mammals are persistent tremor and convulsive seizures. Other neurotoxins such as cyclodiene insecticides and picrotoxin interact with the γ-aminobutyrate (GABA) receptor in neurons. Blockage of the GABA receptor by these antagonists impairs the transport of chloride ions into the cell, which results in partial depolarisation of the membranes in the absence of adequate signals. The clinical symptoms of the pathophysiological changes can be summarized as a state of uncontrolled excitation. In addition, with cyclodiene insecticides this hyperexcitability is augmented by the inhibition of Ca^{2+}–Mg^{2+}–ATPase, which results in accumulation of intracellular free calcium and increased release of neurotransmitters from storage vesicles (Fig. 48).

Organic solvents have a depressant effect on the CNS that results in narcosis by nonspecific alterations in membrane fluidity due to their lipid solubility rather than by interference with specific ion channels. This depressant effect is not based on certain structural features or specific interactions, that is, it is also observed after inhalation of the inert, but highly lipophilic gas xenon.

3.2.3. Interference of Xenobiotics with Oxygen Transport, Cellular Oxygen Utilization, and Energy Production

Continuous, sufficient production of energy in form of adenosine triphosphate (ATP) in specialized cellular organelles (mitochondria) is essential for cell survival and function. The process of cellular energy production requires oxygen, cofactors, and a wide array of specialized enzymes acting in concert. Many chemicals produce their toxic effects by interfering with the transport and cellular utilization of oxygen or with the oxidation of carbohydrates, which is coupled to the synthesis of ATP by oxidative phosphorylation.

The interference with cellular energy production may occur at different sites in the uptake, transport, and cellular utilization of oxygen. Oxygen transport from the lung to tissues may be reduced or blocked by xenobiotics which compete with oxygen for the binding site in the transport protein hemoglobin or which chemically modify this binding site. Carbon monoxide competitively blocks the binding of oxygen to the transport protein hemoglobin. Chemical oxidation of the iron in hemoglobin also impairs oxygen transport. Some xenobiotics oxidize the Fe^{2+} at the oxygen binding site in hemoglobin to Fe^{3+}. Hemoglobin with Fe^{3+}, known as, methemoglobin, can not reversibly bind oxygen. Thus, xenobiotics causing methemoglobinemia (e.g., nitrites, aromatic amines) effectively block oxygen transport.

The cellular utilization of oxygen in the tissues is blocked by cyanide, hydrogen sulfide, and azide because of their affinity for cytochrome oxidase. Cyanide exerts its toxic effects by interrupting electron transport in the mitochondrial cytochrome electron-transport chain. In addition, interferences with the enzyme systems producing ATP is a well-defined mechanism of toxicity. The ultimate formation of ATP in the cell by the oxidation of carbohydrates may also be blocked at other sites. For example, rotenone and antimycin A interfere with specific enzymes in the electron-transport chain, nitrophenols uncouple oxidative phosphorylation, and sodium fluoroacetate inhibits the citric acid (Krebs) cycle. Nitrophenols interfere with the production of high-energy phosphates in mitochondria. They prevent the phosphorylation of adenosine diphosphate (ADP) to ATP, but electron flow and oxygen consumption continue. In addition to inducing loss of cell function due to depletion of ATP, this type of uncoupler also causes a marked elevation of body temperature due to excess heat production. Toxicity resulting from blockage of the tricarboxylic acid cycle occurs in organs heavily relying on the availability of a continuous energy supply and results in cardiac and nervous system toxicity. A classic example for a mechanism inhibiting the Krebs cycle is termed “lethal synthesis” and is exemplified by fluoroacetate, which is incorporated into the Krebs cycle as fluoroacetyl coen-

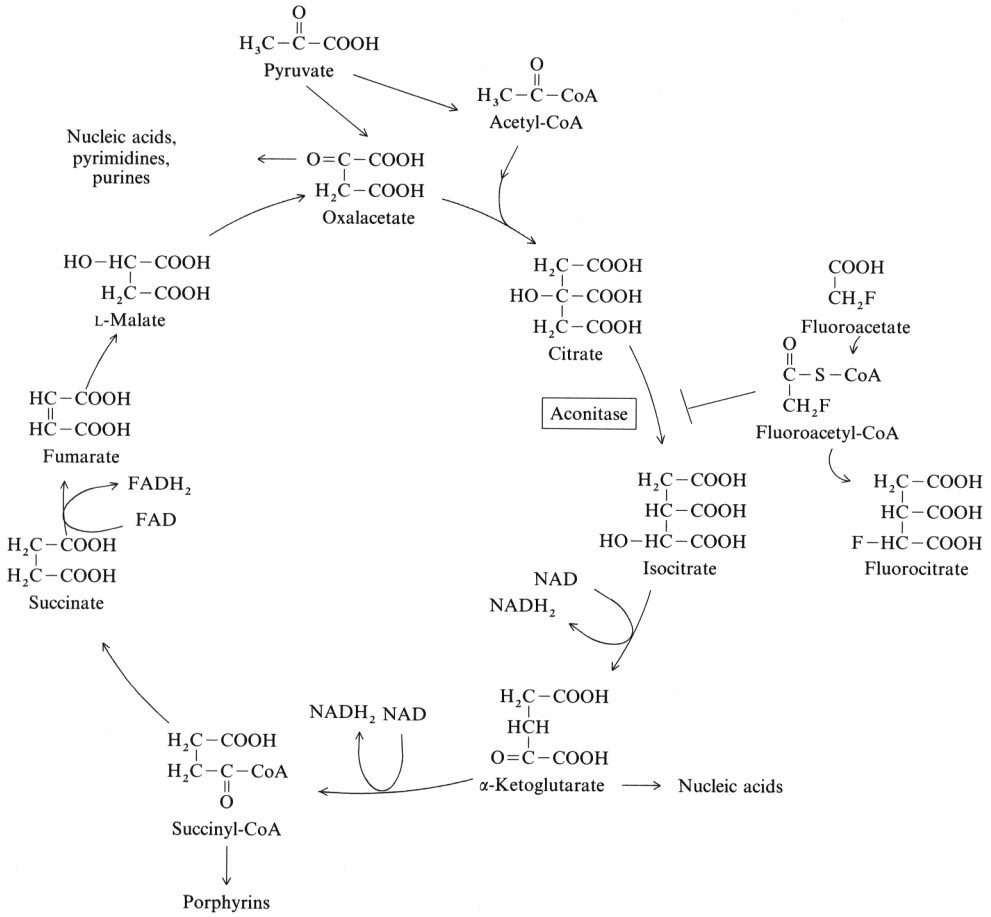


Figure 49. Lethal synthesis
Incorporation of fluoroacetate in the Krebs cycle; formation of fluorocitrate and inhibition of aconitase results in blocking of the tricarboxylic acid cycle and interruption of cellular energy production

zyme A, inhibits the aconitase-catalyzed conversion of citrate to isocitrate, and interrupts the Krebs-cycle (Fig. 49). The Krebs cycle is the major degradative pathway for the generation of ATP and also provides intermediates for biosynthesis. For example, the majority of the carbon atoms in porphyrins comes from succinyl CoA and many of the amino acids are derived from α-ketoglutarate and oxalacetate. Hence, interruption of the Krebs cycle by the incorporation of fluoroacetate may be lethal for the cell.

The consequences of ATP depletion include impairment of membrane integrity, ion pumps, and protein synthesis. Depending on its extent, energy depletion will inevitably Iead to loss of cell function and cell death.

3.3. Binding of Xenobiotics to Biomolecules

Many toxic chemicals exert their effects by covalent linkage of reactive metabolites to essential macromolecules of the cell. Xenobiotics may become covalently bound to the active site of enzymes or to other macromolecules whose function is critical to the cell. These include proteins (Section 3.3.1) and lipids (e.g., as membrane structural elements; see Section 3.3.1 and 3.3.2) and nucleic acids (see Section 3.3.1 and 3.3.2 and 3.3.3). The linkage generally involves binding of electrophilic metabolites to nucleophilic sites such as thiol, amino, and hydroxyl groups in the side chains of proteins and is essentially irreversible; it depends only on the turnover of the

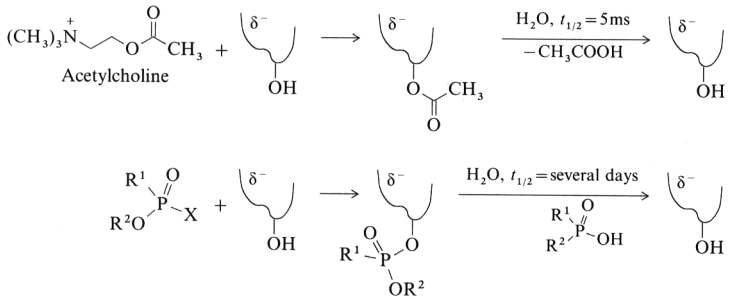


Figure 50. Mechanisms of cholinesterase inhibition by organophosphate pesticides
Compare the $t_{1/2}$ of the enzyme complex with the physiological substrate acetylcholine (a few milliseconds) to the $t_{1/2}$ of the enzyme complex with organophosphate pesticides (several days).

macromolecule in question for the repair of the lesion. In addition, other toxins may bind to proteins and impair their normal function without first being converted to reactive intermediates.

3.3.1. Binding of Xenobiotics or their Metabolites to Cellular Proteins

Many toxic substances exert their effects via binding to the active sites of enzymes or other proteins that are critical to cellular function. For example, hydrogen cyanide binds with high affinity to the Fe^{3+} ion in cytochrome oxidase and thus blocks the last step in the mitochondrial electron-transport chain, which is important for cellular energy production. This single and highly specific site of action is responsible for the rapid induction of the often fatal toxic effects of cyanide. Carbon monoxide binds to the reduced form of iron in hemoglobin, whose physiological function is to bind, transfer, and deliver oxygen to tissues. Since carbon monoxide has a 210-fold higher affinity to this binding site than oxygen, very low concentrations of carbon monoxide in the atmosphere are sufficient to displace the physiological ligand and produce severe toxic effects. Thus, 0.1% carbon monoxide in the atmosphere can occupy roughly 50% of the available hemoglobin binding sites, because on the basis of the 210-fold higher affinity to hemoglobin compared with oxygen, 0.1% carbon monoxide is equivalent to 20% oxygen in the atmosphere.

Another example for the importance of protein binding in toxicity is the binding of metal ions to protein thiol groups. Many toxic metals such as lead, mercury, cadmium, and arsenic bind to proteins with free sulfhydryl groups, which contributes by as-yet largely unknown mechanisms to the toxicity of these metals. Induction of porphyria by lead, mercury, and other metals is based in part on the inhibition of specific enzymes of heme biosynthesis and results in the accumulation of specific intermediates in heme synthesis. The binding of cadmium to the sulfhydryl-rich protein metalothionein results in active concentration of the metal-metalothionein complex in the proximal tubules of the kidney. Catabolism of the metal complex in the lysosomes of proximal tubule cells with concomitant liberation of the toxic metal results in nephrotoxicity, a common effect of cadmium exposure in humans and experimental animals [137].

Binding to active sites in enzymes may result in the inhibition of biochemical pathways vital to the cell. The induction of toxic effects may be due to accumulation of a specific enzyme substrate or insufficient amount of substrate available for normal physiological function. For example, many organophosphate pesticides inhibit cholinesterases by covalently binding to the active site, the amino acid serine of cholinesterase. Since the phosphorylated cholinesterase is stable, the covalent binding results in inhibition of enzymatic activity (Fig. 50). Due to the impaired cleavage, acetylcholine accumulates at cholinergic synapses and neuromuscular junctions. Organophosphates thus produce the typical signs of acetylcholine poisoning, such as increased salivation and lacrimation, abdominal cramps and diarrhea, cough, bronchoconstriction and breathing impairment, mental confusion, headaches, tremor, and coma.

Protein modifications may also be the basis for immunosuppressive effects and chemical-induced allergy. For many chemicals causing necrosis, binding to cellular macromolecules is essential for the expression of toxic effects. However, in most cases the temporal sequence of events that is triggered by covalent binding and the cause–effect relationships between these events are not fully understood.

3.3.2. Interaction of Xenobiotics or their Metabolites with Lipid Constituents

Lipid peroxidation in biological membranes by free radicals initiates a series of events finally causing cellular dysfunction and cell death. The formation of radicals during peroxidation is a self-propagating process; the reaction may be started by organic radicals formed during biotransformation or by oxygen radicals formed by disruption of cellular energy metabolism. The initiation of lipid peroxidation by interaction of free radicals with polyunsaturated fatty acids to form lipid peroxy radicals, which then produce lipid hydroperoxides and other lipid peroxy radicals, has been proposed as a critical step leading to cell injury and death. Peroxidative damage to plasma membrane lipids may cause impairment of membrane integrity and, finally, rupture of the plasma membrane. In addition, breakdown of the membranes of subcellular organelles such as those of the mitochondria, the endoplasmic reticulum, and lysosomes may also contribute to induction of cell death. The end products of the breakdown of membranes, mainly unsaturated aldehydes, may also produce toxicity in distal tissues. Induction of lipid peroxidation is involved in the toxicity of many chemicals which are converted to free radicals. A well-studied example is carbon tetrachloride, which is converted by cytochrome P450 to the trichloromethyl radical (CCl_3) and the trichloromethylperoxyradical (CCl_3O_2). Radicals and reactive oxygen species may interact with the major soluble cellular thiols, glutathione (GSH), and thiol-containing proteins. Depletion of cellular glutathione and modification of thiol-containing proteins by oxidation or mixed disulfide formation with glutathione and other low molecular mass thiols may results in oxidative stress in the cell.

An important function of reduced glutathione is to protect the sulfhydryl groups of proteins by keeping them in the reduced state. Depletion of intracellular glutathione stores appears to be a prerequisite for the onset of significant oxidative stress. Many critical enzymes in the cell depend on reduced thiol groups (SH groups) to maintain their activity; hence, concentrations of reactive oxygen species in excess of that necessary to deplete intracellular glutathione can induce oxidation of protein thiols to form disulfide linkages, thereby impairing enzymatic activity. Although the direct covalent interaction of electrophilic chemicals with protein thiols may contribute to enzyme inhibition; it appears that reversible oxidation of the thiol group as a result of oxidative stress plays a more important role. One group of thiol-containing enzymes whose impairment as a result of oxidative inactivation may play a critical role in cell injury and death are the Ca^{2+}-transporting membrane systems.

3.3.3. Interactions of Xenobiotics or their Metabolites with nucleic Acids

Electrophilic compounds, usually formed by oxidative biotransformation of xenobiotics, may also interact with various nucleophilic sites in DNA, principally O-6, N-7, N-2, and C-2 of guanine. Moreover, other types of DNA damage may occur as a consequence of covalent binding of electrophiles or by interaction of DNA with reactive oxygen metabolites (Table 21).

These interactions may alter gene expression (see Sections 3.5 and 3.7). The changes in gene expression may be quantitative, that is, formation of the wrong amount of a protein in an inappropriate time period of cellular life, or qualitative, that is, formation of a protein with altered or impaired properties. In both cases, the changes may cause the death of the cell. However, interaction of xenobiotics with nucleic acids is more important in generating somatic mutations which can be the initiating event for a process ultimately leading to malignant transformation of cells and tumor growth. Alkylation of the O-6 position of guanine appears important in the mutagenicity and carcinogenicity of nitrosamines and other chemicals that readily form methyl carbonium ions. In addition, other sites such as the N-7, N-2, and C-2 positions of guanine may

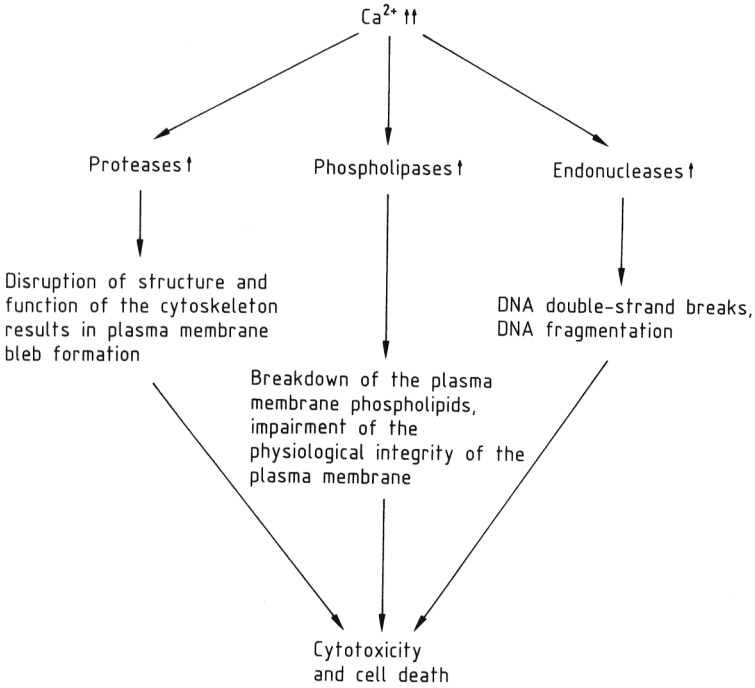


Figure 51. Increased concentrations of intracellular calcium may activate calcium-dependent degradative enzymes, resulting in the destruction of proteins and lipids and in DNA fragmentation, thus causing cell death

also play an important role in DNA adduct formation with other electrophilic chemicals. Ribonucleic acid (RNA) also contains nucleophilic sites, and thus critical intracellular functions of RNA such as protein synthesis may be perturbed by covalent interaction of electrophilic chemicals with RNA.

Table 21. Types of DNA damage induced by xenobiotics

Type of DNA-damage	Examples
Modifications of purines and pyrimidines	alkylation by electrophiles, oxidation to 8-hydroxyguanosine and other oxidized purines, UV-induced formation of thymidine dimers
DNA strand breaks	oxygen radicals, activation of calcium-dependent endonucleases
AP lesions (apurinic, apyrimidinic sites)	loss of DNA bases through labilization after covalent binding of xenobiotics to specific sites in purine and pyrimidines
DNA cross-links	bifunctional electrophiles react with nucleophilic sites in both DNA strands (interstrand cross-link) or with two bases on one strand (intrastrand cross-link)

3.4. Perturbation of Calcium Homeostasis by Xenobiotics or their Metabolites

Intracellular calcium concentrations are rigorously maintained at around 10^{-7} M against an extracellular concentration of more than 10^{-3} M. Three principal buffer systems are important in maintaining this steep gradient in virtually all cells: the plasma membrane, the mitochondria, and the endoplasmic reticulum. All systems require ATP to transport calcium, directly or indirectly. Exposure of freshly isolated or cultured cells to numerous toxins such as *tert*-butyl hydroperoxide, quinones, paracetamol, and carbon tetrachloride induces a rapid and sustained rise in cytosolic calcium concentrations, which correlates well with the subsequent loss of cell viability [138, 139]. Prevention of this rise by omitting calcium in the extracellular buffer or by addition of calcium chelators may prevent cell death. A central event in calcium-induced toxicity is the activation of calcium-dependent degradative enzymes such as proteases, phospholipases, and endonucleases (Fig. 51).

Cytoskeletal integrity is important for a number of cellular functions including motility, shape, secretion, and division. Activation of calcium-dependent proteases results in disruption of cytoskeletal components and formation of plasma membrane blebs (protrusions). In addition, activation of calcium-dependent phospholipases may induce membrane phospholipid breakdown, and calcium-dependent endonucleases may lead to DNA fragmentation by the formation of DNA double-strand breaks. These toxic biochemical cascades may act in concert to cause cell death [104].

3.5. Nonlethal Genetic Alterations in Somatic Cells and Carcinogenesis

The induction of cancer by chemicals is a complex multistep process involving interactions between environmental and endogeneous factors. Tumors are formed as a result of aberrant tissue growth due to loss of control mechanisms of cell division. A model traditionally used for an operational description of carcinogenesis is the initiation–promotion model. Initiation, the first stage, requires a genotoxic event such as binding of an electrophilic xenobiotic to DNA causing a premutagenic lesion in a single cell [140, 141]. After DNA replication, the premutagenic lesion may be transformed into a heritable mutation (i.e. by base-pair substitution; see Section 3.7). In the promotion stage, several, primarily nongenotoxic, mechanisms facilitate (promote) the preferential proliferation of the initiated cell and finally resulting in the formation of a tumor. Initiators are usually genotoxic agents, whereas promoters generally act by interfering with extranuclear sites and processes; most promoters increase cell growth and cell proliferation.

In contrast to initiators, which are believed to result in irreversible changes in the cellular genome without thresholds, promoters show some reversibility, and thresholds for promoters can be discussed. This stepwise nature of carcinogenesis was first shown in mouse skin, and this model has now been well characterized (Fig. 52). In a typical experiment, an initiating chemical such as dimethylbenzanthracene is applied to mouse skin at a low dose so that very few tumors, if any, are produced in the animals lifetime. After an interval of one week to one year, the treated (initiated) skin is exposed to multiple applications of a promoter such as the phorbol esters found in croton oil. Tumors begin to appear within 5 to 6 weeks after application of the promotor, and all mice carry tumors by 10 to 12 weeks after the start of application (Fig. 52).

The initiation–promotion experiments on mouse skin and similar experiments in the rodent liver have led to the following general rules of carcinogenesis:

1) The initiator must be given first; no tumors or very few tumors result if the promoter is given first.
2) The initiator, if administered once at a subcarcinogenic dose, does not produce tumors during the time of observation; however, repeated doses of the initiator may cause tumors even in the absence of the promoter (the initiator is a complete carcinogen in this case).
3) The action of the initiator is irreversible; tumors result in nearly the same yield if the interval between initiation and promotion is extended from one week to one year.

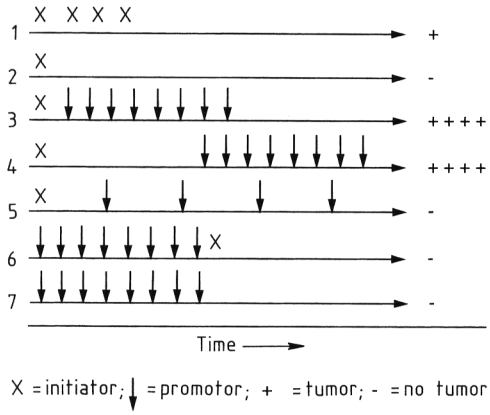


Figure 52. Schematic presentation of the initiation–promotion model of chemical carcinogenesis in the mouse skin
Promoters reduce the latency period and increase the number of tumors only when applied after the initiator (3, 4). The application frequency required for promoting effects to occur depends on the initiating carcinogen and the target tissue. If the application frequency of the promoter is too low, the promoting effects are not observed (i.e., no tumors are formed or the tumor yield is not increased, 5). This is also the case, when the promoter is applied prior to the initiator (6) or alone without initiating agent (7). Tumors can also be produced by the initiating agent alone if it is applied at sufficient concentration or above a certain application frequency (1). In this case, the initiator is termed a complete carcinogen.

4) The initiator is an electrophile, or is bioactivated to an electrophile, which binds covalently to DNA causing a mutation after DNA replication.
5) In contrast, there is no evidence of covalent binding of promoters or their metabolites to DNA.
6) The action or the promoter is reversible at an early stage and usually requires repeated exposure; thus, there is probably a threshold level of exposure to promoters. However, threshold levels can not be reliably defined, as long as the biochemical mechanism involved in tumor promotion is not precisely known. In contrast to the established target of the initiation process (DNA), the molecular mechanisms of tumor promotion are still largely unknown.

In addition to promotion of skin carcinogenesis by the phorbol esters, there are known or suspected promoters for tumors in other organs [142, 143]. Bile acids are known to be promoters of colon carcinogenesis in experimental animals. In humans, there is a strong association between high intake of dietary fat and cancer of the colon; since ingestion of fat increases the amount of bile acids in the colon, the increased incidence of colon cancer may be due to the promoting effect of the bile acids on intestinal epithelia. In rat bladder, saccharin and cyclamate are promoters for tumors initiated by a single dose of dimethylnitrosourea; tryptophane is a promoter for urinary bladder tumors in dogs treated with an initiating dose of 4-aminobiphenyl or 2-naphthylamine. Hormones are also known modifiers of chemical carcinogenesis. Oral or intravenous administration of dimethylbenzanthracene produces mammary tumors in susceptible female mice. Prolactin will increase and accelerate tumor development, whereas ovariectomy results in reduced tumor yield.

In addition to these two stages a third stage, termed progression, has since been established as an integral part of the carcinogenic process. In this last stage additional mutational events increase the malignancy of the tumor, that is, the tumor grows in an invasive manner, destroying the surrounding tissues and forming metastases in other organs (Fig. 53).

The understanding of the molecular mechanisms on route from DNA interactions to a clinically observable tumor requires some knowledge on the basic roles of DNA biochemistry and biology in cellular function and replication. Therefore, a short chapter on DNA biochemistry and biology follows. Further and in-depth information is presented in textbooks on biochemistry and molecular biology.

3.6. DNA Structure and Function

3.6.1. DNA Structure (→ Nucleic Acids, Chap. 2.1)

With the exception of certain viruses, the genetic information of all cells is contained in deoxyribonucleic acid (DNA), whose structure and constituents permit the accurate storage of a vast amount of information. In the cell nucleus, the

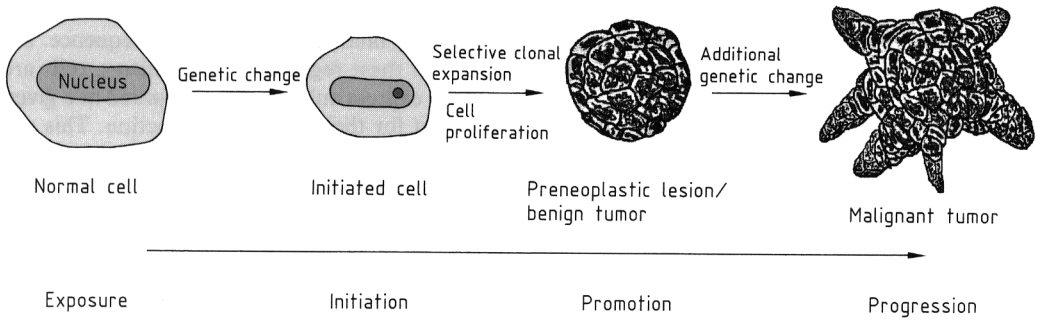


Figure 53. Multistep model of tumor formation
Genotoxic events resulting in heritable mutations cause the formation of an initiated cell (initiation). In the following stage of promotion, nongenetic (epigenetic) events contribute to preferential proliferation of the initiated cell. In the third stage of progression, additional genetic events increase the malignancy of the tumor tissue (i.e., its growth becomes increasingly destructive and metastases are formed in other organs).

DNA is packaged with proteins to form chromatin.

The genetic code of the DNA is denoted by four letters: two pyrimidine nitrogenous bases, thymine (T) and cytosine (C), and two purine bases, guanine (G) and adenine (A). These are are functionally arranged in codons (or triplets). Each codon consists of a combination of three letters and codes for a specific amino acid.

The bases on one strand are connected together by a sugar (deoxyribose) phosphate backbone. DNA can exist in a single-stranded or double-stranded form. In the latter state, the two strands are held together by hydrogen bonds between the bases. The adenine on one strand binds to thymine on the sister strand, and guanine pairs with cytosine. Two hydrogen bonds are involved in the binding between adenine and thymine, while three hydrogen bonds are involved in the binding of guanine to cytosine.

Double-stranded DNA has the unique property that it can make identical copies of itself when supplied with precursors and the required enzymes. In simplified terms, two strands begin to unwind and separate as the hydrogen bonds are broken. This produces single-stranded regions. Complementary deoxyribonucleotide triphosphates then pair with the exposed bases under the control of the enzyme DNA polymerase.

The information in DNA is assembled in structural genes. A structural gene is a linear sequence of codons, which contain the information for a functional protein, consisting of a sequence of amino acids. Individual proteins may function as structural components of the cell, as enzymes, or may regulate important cellular functions. The DNA of eukaryotic cells contains repeated sequences of some genes. Also, eukaryotic genes, unlike prokaryotic (i.e., bacterial) genes, have noncoding DNA regions called introns between coding regions known as exons. This property means that eukaryotic cells have an additional processing mechanism at transcription.

3.6.2. Transcription (→ Nucleic Acids, Chap. 4.2.1)

The linkage between the DNA in the nucleus and proteins in the cytoplasm is not direct (Fig. 54). The information contained in the DNA molecule is transferred to the protein-synthesizing machinery of the cell via another informational nucleic acid, called messenger RNA (mRNA), which is synthesized complementary to the relevant DNA sequence by RNA polymerase. Although similar to DNA, mRNAs are single-stranded, and contain the base uracil instead of thymine, and ribose instead of deoxyribose. The mRNA molecules act as transport vehicles for the information contained in the genes being expressed.

In eukaryotic cells, the initial mRNA copy contains homologues of both the intron and exon regions. The intron regions are then removed and the exon regions are spliced together to form the active mRNA molecules, which are then transported through the pores of the nuclear membrane to the cytoplasm.

3.6.3. Translation (→ Nucleic Acids, Chap. 4.2.2)

The next process involves the translation of mRNA molecules into polypeptides. This procedure requires many enzymes and two further types of RNA: transfer RNA (tRNA) and ribosomal RNA (rRNA). There is a specific tRNA for each amino acids. The tRNA molecules are involved in the transport and coupling of amino acids into the resulting polypeptide (Fig. 54). Each tRNA molecule has two binding sites, one for the specific amino acid, the other containing a triplet of bases (the anticodon) which is complementary to the appropriate codon on the mRNA.

The rRNA is complexed with protein to form subcellular globular organelles called ribosomes. Ribosomes can be regarded as the reading heads, which allows the linear array of mRNA codons each to base-pair with an anticodon of an appropriate incoming tRNA amino acid complex.

3.6.4. Regulation of Gene Expression

All cells possess the same genetic information, but different types of cells exhibit distinct gene transcription patterns. These differences in gene expression are critical to the morphological and biochemical properties of the many thousands of

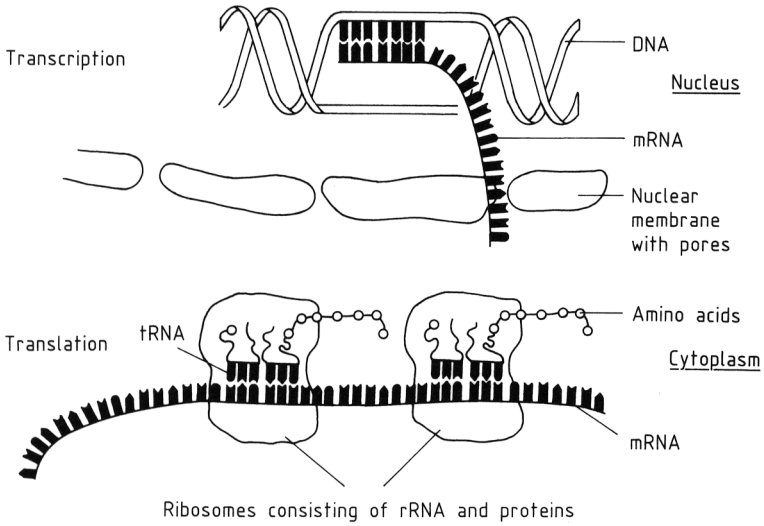


Figure 54. Scheme of the important steps in gene transcription and protein synthesis

cell types of the human and animal body. Hence, mechanisms are required that regulate gene expression, that is, determine which genes are expressed and to what extent and which genes are not expressed in a certain cell type at a particular time. The mechanisms involved in regulation of gene transcription are not entirely understood. The transcription of structural genes is regulated by a special set of codons, in particular, promotor sequences, the initial binding sites for RNA polymerase before transcription begins. Different promoter sequences have different affinities for RNA polymerases. Additional regulatory genes called operators regulate the activity of several genes or gene groups (operons). The activity of the operator itself is further controlled by a repressor protein, which stops the transcription of the whole operon by binding to the operator sequence. Due these regulatory mechanisms cells are able to express only the genes required at a given moment for their specialized function. This not only helps to conserve cellular energy, but is also critical for correct cellular differentiation, tissue pattern formation and function, and maintenance of the physiological integrity of the entire organism.

3.6.5. DNA Repair

All living cells possess several efficient DNA repair processes. DNA repair is crucial in protecting cells from spontaneous and exogenous lethal and mutating effects such as heat-induced DNA hydrolysis, UV radiation, ionizing radiation, DNA-reactive endogenous chemicals, free radicals, and reactive oxygen species. Among the various DNA repair mechanisms, the most comprehensively studied mechanism in eukaryotes is the excision repair pathway. This mechanism involves a group of enzymes acting cooperatively to recognize DNA lesions, remove them, and correctly replace the damaged sections of DNA.

The excision repair pathway is regarded as error-free and does not lead to the generation of mutations. However, this pathway may become saturated after excessive DNA damage. In this case, the cell may be forced to activate other repair mechanisms which do not operate error-free. Several of these mechanisms, such as error-prone repair, have been well characterized in bacteria, but their counterparts, if any, in mammalian cells have not been identified yet.

3.7. Molecular Mechanisms of Malignant Transformation and Tumor Formation

3.7.1. Mutations

Mutations are hereditary changes in genetic information, resulting from spontaneous or xeno-

biotic-induced DNA damage. The term mutation can be applied to point mutations, which are qualitative changes involving one or a few bases within one gene, and to larger changes involving parts of the chromosome detectable by light microscopy or even whole chromosomes and thus many thousands of genes (Table 22).

Table 22. Types of mutations

Gene mutations	base-pair substitutions, deletions, insertions, gene rearrangements, gene amplifications
Chromosomal mutations	
Structural	breaks, translocations
Numerical	loss or gain of an entire chromosome

Point mutations can occur when one base is substituted for another (base substitution) or when base pairs are deleted or inserted (deletions/insertions). Substitution of another purine for a purine base or of another pyrimidine for pyrimidine is called a transition, while substitutions of purine for pyrimidine or pyrimidine for purine are called transversions. Very small alterations in the chemical structure or the DNA bases may be sufficient for a base-pair substitution to occur. Guanine, for example, normally pairs with cytosine, while O^6-methylguanine (a frequent DNA modification seen with methylating agents such as dimethylnitrosamine) pairs with thymine (Fig. 55).

These changes in certain codons may cause insertion of the wrong amino acid into a relevant polypeptide. In this case, the changes are named missense mutations. Such proteins may have dramatically altered properties if the new amino acid is close to the active center of an enzyme or affects the three-dimensional structure of an enzyme or a structural protein. Hence, the alterations may result in marked changes in the differentiation and proliferative characteristics of the affected cells. A base substitution can also result in the formation of a new inappropriate stop (or nonsense) codon. The result of nonsense mutations is the formation of a shorter and, most likely, inactive protein. Owing to the redundancy of the genetic code, about a quarter of all possible base substitutions will not result in amino acid replacement and will be silent mutations.

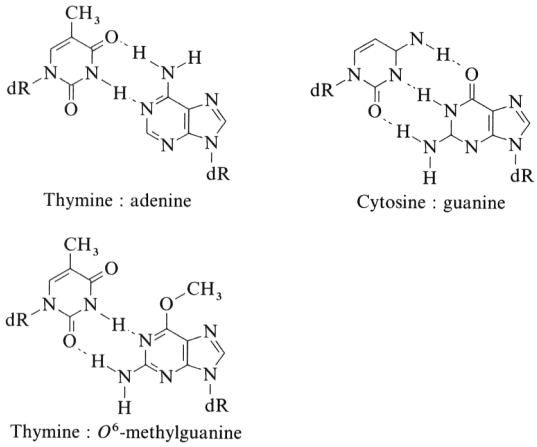


Figure 55. Formation of a base substitution
Guanine normally pairs with cytosine and adenine with thymine (upper part); in contrast, O^6-methylguanine (a frequent DNA modification induced by methylating agents such as dimethylnitrosamine) pairs with thymine (lower part), resulting in a hereditary change of the genetic information

Bases can be also deleted or added to a gene. As each gene is of a precisely defined length, these changes, if they involve a number of bases that is not a multiple of three, result in a change in the reading frame of the DNA sequence and are known as frameshift mutations. Such mutations often have a dramatic effect on the polypeptide of the affected gene, as most amino acids will differ from the point of insertion or deletion of bases in the DNA strand onwards.

Some forms of unrepaired alkylated bases are lethal due to interference with DNA replication. Others, such as O^6-methylguanine lead to mutations if unrepaired. These differences indicate that not all DNA adducts are of equivalent importance. In fact, some adducts appear not to interfere with normal DNA functions or are rapidly repaired, others are mutagenic, and yet others are lethal. The most vulnerable base is guanine, which can form adducts at several of its atoms (e.g., N-7, C-8, O-6 and exocyclic N-2, see Section 2.5.6.6).

Intrastrand and Interstrand Cross-Links. Xenobiotics with bifunctional alkylating properties can also form links between adjacent bases on the same strand (intrastrand cross-links) or between bases on different strands (intrastrand cross-links). The induction of frameshift muta-

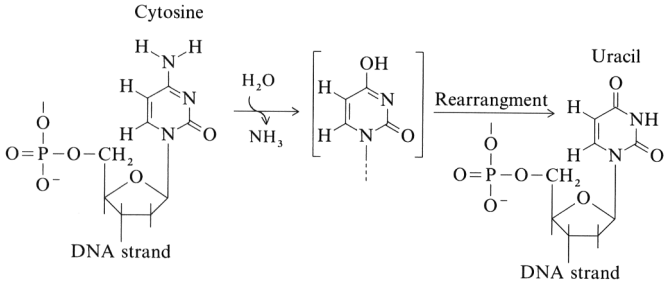


Figure 56. Deamination of cytosine to uracil
dR = Deoxyribose

tion does not necessarily require formation of covalent adducts. Some compounds that have a planar structure, particularly polycyclic aromatic hydrocarbons, can intercalate between the strands of the DNA double strand. The intercalated molecules may interfere with DNA repair or replication and cause insertions and/or deletions of base pairs. The precise molecular event is still unclear, although several mechanisms have been proposed. Hot spots for frameshift mutations often involve sections of the DNA strand taht contain a run of the same base (e.g., the addition of a guanine to a run of six guanine residues).

DNA strand breaks result from the hydrolysis of the sugar–phosphate bond or a nucleotide. In a double-strand, both a single- and double-strand breaks may occur. DNA strand breaks are often induced by hydroxyl radicals, which are formed at high rates both spontaneously during normal cell life and in the presence of exogenous chemicals. AP lesions (apurinic/pyrimidinic sites) in the DNA strand result from spontaneous hydrolysis of the glucosidic bond and loss of the DNA base (see Fig. 37). Similar to the situation with DNA strand breaks, AP lesions are a common spontaneous event; however, the hydrolysis can be dramatically increased by various types of DNA adducts, such as N^7-substituted purines, a common target of alkylating chemicals (see Section 2.5.6.6). Another common spontaneous event is the desamination of cytosine to uracil (Fig. 56); approximately 100 desaminations take place in each cell every day (→ Nucleic Acids, Chap. 4.2.1).

3.7.2. Causal Link between Mutation and Cancer (see also → Carcinogenic Agents; → Mutagenic Agents)

The change from cells undergoing normal, controlled cell division and differentiation to cells that are transformed, divide without control, and are undifferentiated or abnormally differentiated does not occur in a single step. Malignant transformation is a multistage process. Evidence for the involvement of multiple stages comes from in vitro studies, animal models, and epidemiological observations. In humans, the latent period between exposure to a chemical carcinogen and the appearance of a tumor in the target tissue is approximately 10–25 years. Modern molecular-biology techniques enable thorough investigations of the genome of malignant cells in comparison with the genome of their normal counterparts. These studies clearly show that a single mutation is not sufficient to induce malignant transformation. The number of genetic changes varies between two and seven in different tumor types. Also, several types of mutations are usually formed in a malignant transformed cell (i.e., base-pair substitutions, gene rearrangements, chromosomal breaks, and deletions.

3.7.3. Proto-Oncogenes and Tumor-Suppressor Genes as Genetic Targets

Why should mutations be causally linked to cancer? The answer to this question has increasingly become clear since the 1980s with the study of proto-oncogenes and tumor-suppressor genes [144, 145]. It is now appreciated that normal control of cell division and differentiation

is based on the interplay of two sets of genes, the proto-oncogenes and the tumor-suppressor genes. Abnormal activation of proto-oncogenes and/or inactivation of tumor-suppressor genes eventually leads to malignant transformation.

Oncogenes were originally discovered in the genome of transforming retroviruses and were therefore named v-oncogenes [144, 146]. Subsequent studies showed that these viral genes were originally derived from the mammalian genome. In the normal cell, these proto-oncogenes have important functions in signal transduction pathways.

3.7.4. Genotoxic versus Nongenotoxic Mechanisms of Carcinogenesis

Oncogene activation and tumor-supressor gene inactivation induced by mutations provide strong evidence for the involvement of genotoxic mechanisms in tumor formation. However, it has been recognized for many years that cancers can arise without direct or indirect interaction between a chemical and cellular DNA, that is, in the absence of direct mutations. The distinction between nongenotoxic and genotoxic carcinogens was more sharply defined following the identification of a comparatively large number of nongenotoxic carcinogens by the U.S. National Toxicology Program [147]. These include a wide range of chemicals acting by a variety of mechanisms, such as disruption of normal hormonal homeostasis in hormone-responsive tissues, and peroxisome proliferation and proliferation of urothelial cells of the urether and urinary bladder following damage by kidney stones.

Genotoxic carcinogens tend to induce tumors in several tissues of both males and females in both rats and mice. In contrast, nongenotoxic carcinogens usually induce tumors only at high doses, in one tissue, in one sex, or only in one species. The experimental evidence available so far does not support the existence of real thresholds for DNA-reactive carcinogens, although very low concentrations may exist for which practically no clinically manifest tumors may be observed in the animal (or human) lifespan. However, these concentrations can not be considered as thresholds. In the case of carcinogens that operate via other biological effects, the carcinogenic activity would parallel dose–response relationships of the relevant biologic effects, a very important aspect for human risk assessment. Treatment regimens or exposure scenarios that do not elicit biological effects would not promote tumor formation. There are, however, two major problems: very few dose–response studies have been performed with nongenotoxic carcinogens, and in most cases the biochemical mechanisms responsible for the tumor promoting action are not understood.

Usually, non-enotoxic carcinogens are divided into two major categories. The first includes compounds that induce cytotoxicity and regenerative cell proliferation, e.g., 2,2,4-trimethylpentane and other branched-chain hydrocarbons in the proximal tubules. The second group of nongenotoxic carcinogens induce cell proliferation in the absence of cytotoxicity (they are directly mitogenic), relevant examples in this group are carcinogenic hormones or peroxisome proliferators such as di(2-ethylhexyl) phthalate and clofibrate. Induction of cell proliferation is involved in both categories of nongenotoxic carcinogens and may contribute to malignant transformation by increasing the number of spontaneous genetic errors, since DNA replication does not occur with 100% fidelity. Furthermore, in rapidly proliferating cells, DNA damage has a higher chance of being converted to heritable mutations. However, in all these cases cell proliferation is the final result of an as-yet unidentified molecular mechanism.

Genotoxic and nongenotoxic mechanisms are not mutually exclusive events. Rather, they cooperate in tumor formation, as can be seen with many genotoxic carcinogens. In most cases genotoxic carcinogens induce tumors only after applications of high doses, concomitantly causing cytotoxicity, cell death, and regenerative proliferation. Hence, a tumor is the final outcome of a complex, multistep interplay between genotoxic and extranuclear events.

3.8. Mechanisms of Chemically Induced Reproductive and Developmental Toxicity

The term reproductive toxicity covers any detrimental effect on the male and female reproductive system due to exposure to toxic chemicals.

Developmental toxicity refers to detrimental effects produced by exposure to developing organisms during embryonic, fetal, and neonatal stages of development. The main phases of reproduction are listed below:

Germ cell production
Spermatogenesis (man)
Oogenesis (woman)

Preimplantation phase
Fertilization
Formation of the blastocyst
Implantation

Embryonic phase
Organogenesis (in humans the first 12 weeks, in rat the first 2 weeks of gestation)

Fetal phase
Functional maturation and growth of the organs, in humans week 12 to 30

Peri- and postnatal phase
Last week of gestation, birth and first period after birth

Such effects can be irreversible or reversible. Embryolethal effects are incompatible with survival and result in resorption or spontaneous abortion. Irreversible effects that are compatible with survival may cause structural or functional abnormalities in the offspring, and these are called teratogenic. Embryotoxic chemicals may also cause overall growth retardation or delayed growth of certain organs.

3.8.1. Embryotoxicity, Teratogenesis, and Transplacental Carcinogenesis

For an agent to be classified as a developmental toxicant, it must be harmful to the developing organism at exposure levels that do not induce severe toxicity in the mother, such as substantial reduction in weight gain, persistent emesis, or convulsions. Adverse effects on the developing organism under severe maternal toxicity may be secondary to perturbations in the maternal system. For practical purposes, however, the test compounds can be initially administered at maternally toxic doses to determine the threshold level for adverse effects on the offspring. At these exposure levels conclusions can be qualified to indicate that adverse effects of the conceptus were obtained at maternally toxic exposure levels, and may not be indicative for selective developmental toxicity.

The susceptibility of the developing organism to xenobiotic insults varies dramatically within the narrow time span of the major developmental stages (the preimplantation, embryonic, fetal, and perinatal periods), because developing organisms undergo rapid and complex changes within this short period. The major morphogenic events occuring during preimplantation development are formation of a compact mass of cells (the morula) and of the blastocyst. The latter already exhibits a certain degree of cellular differentiation. Considerable similarity exists in the timing of preimplantation development across several mammalian species, regardless of the total length of gestation [148]. At the time of blastocyst formation, cell division and metabolic capacity increase dramatically. During the preimplantation period, biochemical changes under progesterone and estrogen control render the endometrium sensitive to the blastocyst implantation. One important sign of blastocyst implantation is a prostaglandin-dependent increase in endometrial vascular permeability. Alterations in the hormonal milieu or direct excretion of specific xenobiotics into the uterine epithelia during this period can impair implantation and cause embryolethality. Limited data suggest that the preimplantation embryo appears to be susceptible to lethality but rarely to induction of structural aberrations (teratogenicity) with chemical insults. Sublethal exposures of preimplantation embryos have not yet been adequately explored.

Following implantation, organogenesis takes place, which is characterized by the division, migration, and association of cells into primitive organs. The most characteristic susceptibility of the embryo to xenobiotics during the organogenesis period is the induction of structural birth defects (terata). Within the organogenesis period (embryonic period), individual organ systems possess highly specific periods of vulnerability to teratogenic insult (Fig. 57). Administration of a teratogen on day 9 of rat gestation would result in a high level of brain and eye defects, while on day 15 structural abnormalities of the kidney and urinary bladder would predominate. As shown in Figure 57, critical periods for

the susceptibility of different organs overlap, so that exposure to teratogens usually results in a spectrum of more or less severe malformations in a number of organ systems [149].

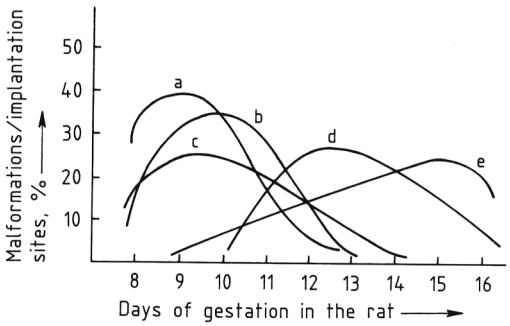


Figure 57. Degree of susceptibility of various organ rudiments of the developing organism to teratogenic xenobiotics (data from [144])
a) Eye; b) Brain; c) Heart and axial skeleton; d) Palate; e) Urogenital system

The critical phase for inducing anomalies in individual organ systems may be as short as one day or may extend throughout organogenesis. Urogenital defects, for example, can result from drug treatment from day 9 to 16 of gestation in the rat. However, the structural defects of the urogenital system depend upon the day(s) of exposure to the teratogenic antibiotic. The development of the urogenital system is multiphasic, and individual stages may have different sensitivities to chemical insult. The mechanisms governing embryonic differentiation are not well understood, but they are certainly involved in intrinsic susceptibility of individual organs to teratogenic insult.

Functional maturation and growth are the major processes occurring after organogenesis, during the fetal and perinatal periods. Insult at these late developmental stages leads to growth retardation or to more specific functional (but not structural) disorders and transplacental carcinogenesis. The fetal and perinatal period of life is highly susceptible to carcinogenesis, due to the high cellular replication rates, presence of xenobiotic biotransforming enzymes in the fetus, and immaturity of the immune system in the developing organism. Several childhood tumors occur so early after birth that prenatal origin is considered likely. These include acute lymphocytic leukemia, Wilms tumor (nephroblastoma), and neuroblastoma. Studies with direct-acting transplacental carcinogens such as ethylnitrosourea indicate that susceptibility to carcinogens begins after completion of the organogenesis period in rodents. Tumors in offspring occurred primarily when ethylnitrosourea was given during the fetal period, whereas birth defects and embryolethality predominated with exposures in the embryonic phase [150, 151]. However, this does not imply that teratogenesis and carcinogenesis are mutually exclusive processes. Teratogenesis and carcinogenesis can be regarded as graded responses of the embryo to injury, with teratogenesis representing the more gross response involving major tissue necrosis in early, relatively undifferentiated embryos, combined carcinogenicity–teratogenicity damage in older embryos, and finally, carcinogenicity alone in the fetus.

3.8.2. Patterns of Dose–Response in Teratogenesis, Embryotoxicity, and Embryolethality

The major toxic effects of prenatal exposure observed at the time of birth are embryolethality, malformations, and growth retardation. The relationship between embryolethality, malformations, and growth retardation is quite complex and depends on the type of agent, the time of exposure, and the dose. Some developmental toxins may cause malformations of the entire litter at exposure levels that do not cause embryolethality (Figure 58). If the dose is increased, embryolethality can occur, often in combination with severe maternal toxicity. Malformed fetuses are often more or less retarded in growth, and the curve for growth retardation is often parallel to and slightly displaced to the right from the curve for teratogenicity. Such a pattern of response is indicative of agents with high teratogenic potency.

A more common dose–response pattern involves embryolethality, malformations, and growth retardation of surviving fetuses. Exposure to these chemicals results in a combination of resorbed, malformed, growth-retarded, and "normal" fetuses. Depending on the teratogenic potency of the agent, lower doses may cause predominantly malformations. As the dosage increases, however, embryolethality predominates until the entire litter is resorbed. Growth retarda-

tion can precede both these outcomes or parallel the teratogenicity curve.

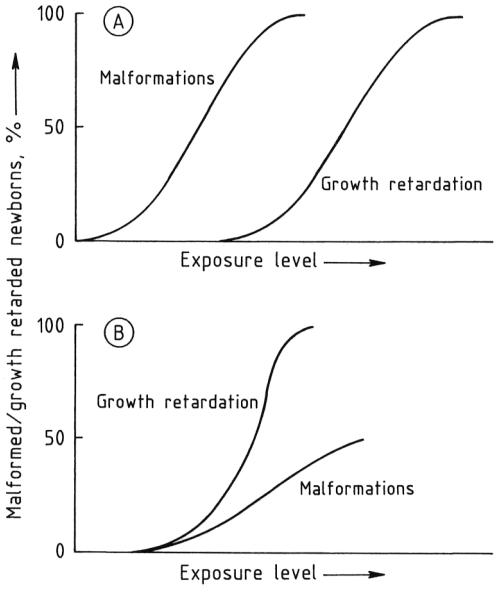


Figure 58. Possible dose – response relationship of teratogens.
A) Teratogens interfering with specific events in differentation; B) Teratogens acting via general cytotoxicity and induction of cell necrosis

A third dose–response pattern consists of growth retardation and embryolethality without structural abnormalities. Growth retardation of surviving fetuses usually precedes significant embryolethality. Agents producing this pattern of response would be considered embryotoxic but not teratogenic, and are also toxic to the maternal organism. In contrast, potent and specific teratogens often show only weak toxicity to the maternal organism.

The best known example is the hypnotic sedative thalidomide, which was rarely associated with severe undesired effects in adult humans but induced malformation in thousands of children whose mothers had taken the drug as a sleeping aid at the recommended therapeutic doses during gestation. The existence of these three general patterns of response indicate that for some agents embryolethality and teratogenicity are different degrees of manifestations of the same primary insult. For other agents, there is a qualitative difference in response, and the primary insult leads to embryotoxicity and embryolethality alone.

For practical purposes (for exact experimental procedures, see Section 4.7.2) a relatively small number of pregnant rodents (approximately eight per group) are exposed on days 6 through 15 of gestation to the test agent at doses up to those causing limiting maternal toxicity and/or severe embryotoxicity (death, severe growth retardation). The purpose of this dose-range finding study is to obtain a qualitative yes/no signal about the potential developmental toxicity of the agent, and information on doses causing severe impairment of the maternal organism. For the main evaluation of developmental toxicity the highest dose should cause measurable but slight maternal toxicity (i.e., significant depression of weight gain) or embryotoxicity (i.e., significant depression of fetal body weight, increased embryolethality, and/or structural malformations), and the low dose should cause no observable effects.

4. Methods in Toxicology

4.1. Toxicological Studies: General Aspects

The aim of toxicology is the assessment and management of potential hazards from exposure of humans and the general environment including animals and plants to chemicals. To achieve this objective, detailed knowledge on the inherent hazard of a xenobiotic (for definition, see Section 5.2.1), that is, its acute and chronic toxicity, its no observed effect level (NOEL), and its teratogenic, mutagenic and carcinogenic effects, is required. This information can not be obtained from a single experiment. A battery of in vivo and in vitro toxicity tests must be utilized. As required by law in most industrialized countries, all toxicity testing must be performed under the rules of good laboratory practice with exact documentation of all relevant conditions and results. Adequate planning of toxicity tests for obtaining optimal information from the experiments may greatly improve the basis for the risk assessment of a chemical and may reduce the number of animals needed and the financial expense associated with toxicity studies. Therefore, all test batteries should be part of an integrated approach to toxicity studies and include not only methods to determine the toxic effects of a chemical

Table 23. OECD guidelines on short- and long term toxicity testing in vivo

No.	Title	Original adoption	Updated
401	Acute Oral Toxicity	12 May 1981	20 Dec. 2002*
402	Acute Dermal Toxicity	12 May 1981	24 Feb. 1987
403	Acute Inhalation Toxicity	12 May 1981	
404	Acute Dermal Irritation/Corrosion	12 May 1981	24 April 2002
405	Acute Eye Irritation/Corrosion	12 May 1981	24 April 2002
406	Skin Sensitization	12 May 1981	17 July 1992
407	Repeated Dose 28-Day Oral Toxicity Study in Rodents	12 May 1981	27 July 1995
408	Repeated Dose 90-Day Oral Toxicity Study in Rodents	12 May 1981	21 Sept. 1998
409	Repeated Dose 90-Day Oral Toxicity Study in Non-Rodents	12 May 1981	21 Sept. 1998
410	Repeated Dose Dermal Toxicity:28-Day	12 May 1981	
411	Subchronic Dermal Toxicity: 90-Day	12 May 1981	
412	Repeated Dose Inhalation Toxicity: 28/14-Day	12 May 1981	
413	Subchronic Inhalation Toxicity: 90-Day	12 May 1981	
414	Prenatal Developmental Toxicity Study	12 May 1981	22 Jan. 2001
415	One-Generation Reproduction	Toxicity	26 May 1983
416	Two-generation Reproduction Toxicity Study	26 May 1983	22 Jan. 2001
417	Toxicokinetics	4 April 1984	
418	Delayed Neurotoxicity of Organophosphorus Substances Following Acute Exposure	4 April 1984	27 July 1995
419	Delayed Neurotoxicity of Organophosphorus Substances: 28-Day Repeated Dose Study	4 April 1984	27 July 1995
420	Acute Oral Toxicity – Fixed Dose Procedure	17 July 1992	17 Dec. 2001
421	Reproduction/Developmental Toxicity Screening Test	27 July 1995	
422	Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test	22 March 1996	
423	Acute Oral Toxicity–Acute Toxic Class Method	22 March 1996	17 Dec. 2001
424	Neurotoxicity Study in Rodents	21 July 1997	
425	Acute Oral Toxicity: Up-and-Down Procedure	21 Sept. 1998	17 Dec. 2001
426	Developmental Neurotoxicity Study	Draft New Guideline, October 1999	
427	Skin Absorption: In vivo method	Expected, Approved by WNT (May 2002)	
428	Skin absorption: In vitro method	Expected, Approved by WNT (May 2002)	
429	Skin Sensitization: Local Lymph Node Assay	24 April 2002	
430	In Vitro Skin Corrosion: Transcutaneous Electrical Resistance Test (TER)	Expected, Approved by WNT (May 2002)	
431	In Vitro Skin Corrosion: Human Skin Model Test	Expected, Approved by WNT (May 2002)	
432	In Vitro 3T3 NRU phototoxicity test	Expected, Approved by WNT (May 2002)	
433	Acute Inhalation Toxicity: Fixed Dose Procedure	Draft New Guideline, October 1999	
451	Carcinogenicity Studies	12 May 1981	
452	Chronic Toxicity Studies	12 May 1981	
453	Combined Chronic Toxicity/Carcinogenicity Studies	12 May 1981	

*Date of deletion.

and their dose dependence, but also toxicokinetics, biotransformation, and mechanisms of action. This chapter provides an overview of the currently used methods for the assessment of a chemical's toxic profile. For details, specific guidelines on practical aspects of toxicity studies and types of data required can be obtained from web sites of national and international organisations (see Section 5.2.1 and 1.4). OECD guidelines for the toxicity testing of chemicals in vivo are listed in Table 23.

For the evaluation of a new chemicals toxic effects in laboratory animals two types of studies are carried out: acute-toxicity and repeated-dosing studies.

Acute Toxicity. Following administration of a single dose of the test substance or of multiple doses given over a period of up to 24 h, potentially adverse effects are usually monitored during the following 14 d. Acute toxicity studies in animals aim to assess the human risk from single exposure to high doses, for example, in industrial accidents, after drug overdoses, or after suicide attempts.

Repeated-Dosing Studies: Subacute, Subchronic, and Chronic Toxicity. The purpose of repeated daily doses of a chemical for part of the animal's life span is to study subchronic and chronic effects. Studies on *subacute toxicity* are carried out for two to four weeks, while studies

on *subchronic toxicity* usually last for a period of three months. These studies are helpful in assessing the human risk resulting from frequent exposure to household or workplace chemicals and from intake of chemicals used for therapeutic purposes. Studies to determine *chronic toxic effects* are carried out for at least six months; studies aiming to investigate the carcinogenic effects of a test compound are carried out over the animal's entire lifetime. Lifetime exposure of humans may occur to widespread environmental pollutants, food additives, or residues of agricultural chemicals in food.

In addition to the acute and repeated-dose toxicity studies, the reproductive and developmental toxicity as well as the genotoxicity of a new chemical must be investigated in separate experiments.

Many thousands of new and potentially toxic compounds are synthesized every year. It would be a waste of money, resources and manpower if the entire battery of toxicity tests were automatically performed for every new chemical. Therefore, toxicity testing is rather undertaken on the basis of a decision-point approach in several stages, as shown in Figure 59. At the end of every stage, the decision must be met, if the development will be continued or if, on the basis of the toxicity data available so far, the potential human risk of the exposure to this chemical is unacceptable. If the latter is true, the development and consequently the toxicity testing is stopped.

Animal Husbandry. The use of standardized conditions for the housing of animals plays a major role in the planning, evaluation, and interpretation of toxicity tests. Animals must be kept in a controlled environment, i.e., constant temperature of 22 ± 3°C, sufficient ventilation, relative humidity between 30 and 70% and a 12 h light/dark cycle. Diet composition and quality of drinking water must also be standardized and controlled throughout the experiment. Only healthy young adult animals should be enrolled in the studies, and the animals should be allowed to acclimatize to the experimental conditions for at least one week prior to first dosing. After the acclimatization period, animals with poor health or body weights varying by more than 20% of the the group's mean body weight are either excluded from the studies or randomized to ensure a homogenous population in the different control and treatment groups. The basic guidelines – choice of species, number of animals, dosing regimens, duration and frequency of observation, assessment of specific body functions – for acute, subchronic and chronic toxicity tests are summarized in Table 24.

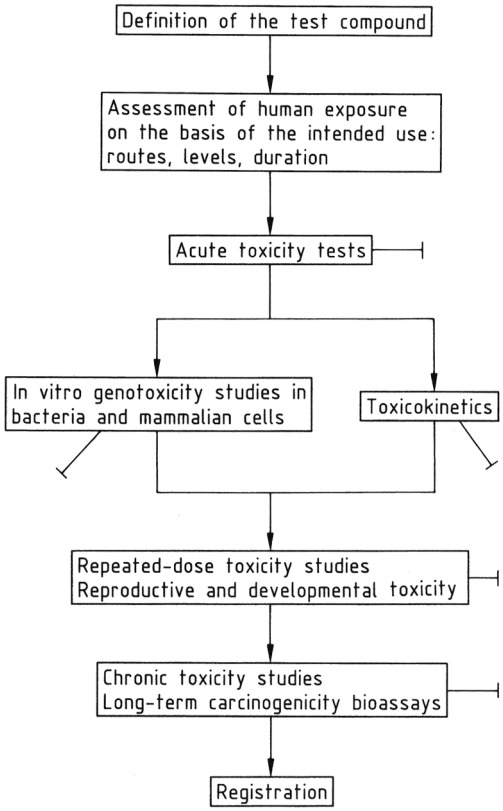


Figure 59. Evaluation of the toxicity profile of a new chemical compound on the basis of a decision point approach At every step of toxicity testing, further development may be interrupted if, on the basis of data collected up to that point, the human risk is considered unacceptable.

A variety of in vitro methods are in development to reduce the numbers of animals used in toxicity testing. Some of the developed methods have gained regulatory acceptance, and some may be used for a priority-determining process for further testing. All well-evaluated methods focus on local effects such as skin and eye irritation, where most progress in the development of nonanimal methods has been made. Regarding replacement of toxicity studies on systemic effects after repeated exposure by nonanimal

Table 24. Basic guidelines for acute, subchronic, and chronic oral toxicity tests

	Acute oral	Subchronic oral	Chronic oral
Animals	rats preferred	rodent and nonrodent species	rodent and nonrodent species
Sex	males and females equally distributed per dose level		
Age	young adult, weight variation within 20 % of mean	rodents, 6 weeks; dogs 4 – 6 months old	
Number of animals	at least 10 (5 per sex)	at least 20 for rodents (10 per sex)	50 per sex group for rodents
Number of treatment groups	3; mortality rates between 10 and 90 % should be produced	3; mortality should not exceed 10 % in high-dose group	3; low dose should reflect expected human exposure and high dose must produce not more than 10 % mortality
Untreated control	not necessary	yes	yes
Vehicle control	yes, if vehicle of unknown toxicity is used	yes	yes
Dosing	gavage; single dose, same dose of vehicle; if necessary use divided doses over 24 h	diet, gavage, drinking water	diet, gavage, drinking water
Duration of study	at least 14-d observation period	90 d	6 – 24 months in rats
Body weight determination	before dosing, weekly thereafter, and at death	weekly and at termination	weekly for first 13 weeks; every 2 weeks thereafter and at termination
Necropsy	all animals	all test animals; organ weights of liver, kidney, heart, lungs, brain, gonads, adrenals, and spleen	
Histopathology	examination of organs showing evidence of gross pathological change	all tissues high-dose and control groups; liver, kidney, heart, lungs, target organs, and any gross lesion in mid- and low-dose groups	all tissues of animals
Frequency of cage-side observations	frequently during day of dosing; once each morning and late afternoon thereafter	daily	daily
Observations, assessments	nature, onset, severity, and duration of any effect observed		
		ophthalmoscopy: pretest and at termination in control and high-dose groups	
		hematology/clinical chemistry:	
		pretest, dosing midpoint, termination	pretest and at 3, 6,12, 18, 24 months
		urine analysis: dosing midpoint, termination	pretest and at 3, 6,12, 18, 24 months

methods, due to the complexities of interactions resulting in toxic responses, acceptable nonanimal tests equaling the predictive power of animal testing are unlikely to be available in the near future.

4.2. Acute Toxicity

4.2.1. Testing for Acute Toxicity by the Oral Route: LD_{50} Test and Fixed-Dose Method

The objectives of acute toxicity tests are

1) To assess the intrinsic toxicity of the test compound
2) To identify target organs of toxicity affected by the xenobiotic
3) To provide information concerning the dose selection and treatment regimens for repeated-dose studies
4) To provide information for human risk assessment after a single high-dose exposure to the chemical
5) To provide essential data for the classification, labeling, and transportation of the chemical (regulatory view report)

LD_{50} Test. The determination of the mean lethal dose (LD_{50}) is still often considered as the first step in the evaluation of the acute oral or inhalation toxicity of a new chemical; with the present knowledge and recent experiences, the formal determination of the LD_{50} is no longer considered as necessary, and alternative methods that are also to be used for classification and labeling have been developed. However, since the test is still widely used, it will be briefly described before treating the newer methods used in testing of acute toxicity and the reasons that led to these changes.

In the LD_{50} test, groups of animals (usually female rats) are treated with graduated doses of the test compound, and by using mathematical models the dose which causes death in 50% or more of the population is determined. Internationally accepted guidelines recommend the use of at least three dose groups with five males and five females for each dose or the use of three dose groups with five animals of one sex and one dose with five animals of the other sex [152]. The LD_{50} is then determined from data obtained as described in Section 1.7. Chemicals with LD_{50} values ≤ 25 mg/kg are considered very toxic, between 25 and 200 mg/kg as toxic, and between 200 and 2000 mg/kg as harmful. Some potentials and limitations of the LD_{50} test follow:

Potential

1) Useful as a first approximation of hazards in the workplace
2) Basis for the design of subchronic studies
3) Properly conducted test may give useful information on other relevant toxicity parameters
4) Rapid completion

Limitations and Problems

1) Lethality only criterion applied, other toxic effects not considered
2) Animal welfare is major point of concern because large number of animals are required to obtain statistically acceptable values
3) Large variations in LD_{50} in different laboratories with identical chemicals, many influencing factors
4) Species and strain differences cause difficulties in extrapolation
5) No information on chronic toxicity obtained (chronic effects are more important for regulating exposure)

The scientific significance of the LD_{50} test has been repeatedly questioned, not only because the lethal dose is not relevant for human risk assessment but also on the basis of the variability of the test results and last not but least for reasons of animal welfare. Comparative assessment of LD_{50} values in 60 laboratories under controlled conditions resulted for example in considerably different LD_{50} values (by a factor of up to 14). Therefore, numerous alternatives to the LD_{50} test have been proposed for the evaluation of acute toxicity that rely on signs of toxicity rather than on mortality. One of this procedures, the fixed-dose method – has recently gained acceptance by the OECD and the EU.

Fixed-Dose Method. The fixed-dose method relies on the observations of clear signs of toxicity developed at one of a series of fixed-dose levels (i.e. 5, 50, 300, and 2000 mg/kg of the chemical oper kilogram body weight). The dose levels at which signs of toxicity but no deaths are detected are used to classify the test compounds according to their toxic potential (Table 25).

Table 25. Classification of toxicity of a xenobiotic with the fixed-dose method

Dose (oral), mg/kg	Results	Classification
5	less than 90 % survival	very toxic
	90 % or more survival but evident toxicity	toxic
	90 % or more survival, no evident toxicity	retest at 50 mg/kg
50	less than 90 % survival	toxic, retest at 5 mg/kg
	90 % or more survival, but evident toxicity	harmful
	90 % or more survival, no evident toxicity	retest at 500 mg/kg
500	less than 90 % survival or evident toxicity and no death	harmful, retest at 50 mg/kg
	no evident toxicity	retest at 2000 mg/kg
2000	less than 90 % survival	harmful
	90 % or more survival, with or without evident toxicity	unclassified, does not represent a significant acute toxic risk if swallowed, no further testing necessary

As can be seen in Table 25, the fixed-dose method allows a classification identical to that previously obtained in the LD_{50} test. Moreover, comparative investigations utilizing both the LD_{50} test and the fixed-dose procedure revealed that in the majority of the test compunds (80–90%), the toxicity class assigned by determining the LD_{50} was identical to that determined by the fixed-dose method [152, 153].

In the fixed-dose method, at least ten animals (five per sex) are used for each dose investigated. The initial dose chosen (5, 50, 300, or 2000 mg/kg body weight) is one that is judged likely to produce evident toxic effects, but no mortality. When such a judgement can not be made due to lack of information on the potential toxic effects of the xenobiotic, an initial "sighting" study should be carried out. If clear signs

of toxicity do not occur at the starting dose of 300 mg/kg during the two weeks observation period, the dose is increased to the next level. A careful clinical examination of the animals is performed at least twice on the day of administration and once daily thereafter for the next two weeks. Animals obviously in pain or showing severe signs of distress or toxicity are humanely killed. Cage-side examinations include skin and fur, eyes and mucous membranes, respiratory system, blood pressure, somatomotor activity, and behavior (for procedures, see Section 4.11). Particular attention is directed to observation of tremors, convulsions, hypersalivation, diarrhoea, and coma as indices of neurotoxicity. Food consumption and weight development are also monitored constantly. At the end of the observation period, all animals in the study are killed and subjected to gross autopsy. Organs showing macroscopic evidence of gross pathology are further subjected to histopathological examination.

The fixed-dose method offers several important advantages as compared with the traditional LD_{50} test:

1) The available evidence suggest that the fixed-dose method produces more consistent results without substantial interlaboratory variation.
2) It provides information on the type, time of onset, duration, and consequences of toxic effects. This information is more relevant for assessing the risks of human exposure to the chemical than the mean lethal doses of the LD_{50} test.
3) It requires fewer animals than the LD_{50} test (roughly 50%) and subjects the animals to less pain and distress.
4) It enables the classification of chemicals according to regulatory requirements.

For the "standard" acute oral and dermal tests the LD_{50} should be determined, except when the substance causes no mortality at the limit dose (usually 2000 mg/kg). Similarly, for an acute inhalation toxicity study the LC_{50} should be determined, unless no mortality is seen at the limit concentration (5 mg per L per 4 h for aerosols and particulates, 20 mg per L per 4 h for gases and vapors). In the fixed-dose procedure, the discriminating dose (the highest of the preset dose levels which can be administered without causing mortality) should be determined. For the acute toxic class and the up-and-down methods the final dose used in the study should be determined following the testing protocol, except when the substance causes no mortality at the limit dose.

Whichever approach is used in determining acute toxicity critical information must be derived from the data to be used in risk assessment. It is important to identify the dose levels at which signs of toxicity are observed, the relationship of the severity thereof with dose, and the level at which toxicity is not observed (i.e. the acute NOAEL). However, note that a NOAEL is not usually determined in acute studies, partly because of the limitations in study design.

4.2.2. Testing for Acute Skin Toxicity

Irrespective of whether a substance can become systemically available, it may cause changes at the site of first contact (skin, eye, mucous membrane/gastrointestinal tract, or mucous membrane/respiratory tract). These changes are considered local effects. A distinction can be made between local effects observed after single and after repeated exposure. For local effects after repeated exposure, see Section 3.9. Only local effects after single ocular, dermal, or inhalation exposure are dealt with in this section. Substances causing local effects after single exposure can be further classified as irritant or corrosive substances, depending on the (ir)reversibility of the effects observed.

Irritants are noncorrosive substances which through immediate contact with the tissue can cause inflammation. Corrosive substances are those which can destroy living tissues with which they come into contact.

Knowledge on the dermal toxicity of a new chemical is one of the prerequisites for assessment of the risks associated with human exposure to the chemical, because skin contact may represent a very important route of exposure in the occupational setting and in the home. Testing for dermal toxicity is usually performed in rabbits. Three types of application of the test chemical are employed: nonocclusive, semiocclusive, and occlusive. The test compound is applied uniformly to the back or a band around the trunk (clipped free of hair); approximately

10% of the body surface of the animal should be covered. Solid substances are pulverized and moistened to a paste with physiological saline or another appropiate solvent whose effects have been fully evaluated prior to the skin test. For occlusive or semiocclusive testing, the application site is covered with a plastic sheet (or other impervious material) or with a porous gauze dressing, respectively. For unocclusive exposure, the application site should be as close to the head as possible to prevent ingestion of the chemical by the animal licking the site of application. The duration of exposure varies between 4 and 24 h. If no test-chemical-related toxic effects on the skin or systemic toxicity are observed up after doses of up to 2 g/kg body weight, testing at higher doses is unnecessary. At the end of the exposure period, the compound is removed with cotton wool soaked in an appropiate solvent and the skin irritation is scored according to the Draize scoring system as shown in Table 26. In addition, any adverse systemic effects caused by percutaneous absorption of the test compound are monitored.

However, substantial differences exist in skin anatomy between humans and experimental animals. In general, the penetration of chemicals through the human skin is similar to that of pig, miniature swine, and squirrel monkey and clearly slower than that of the rat and rabbit. For example, administration of the insecticides lindane and parathion to rabbit skin results in an absorption of 51.2 and 99.5% of the dose, respectively; the corresponding absorption rates for human skin are 9.3 and 9.7%.

Since the 1980s, in vitro studies using human skin samples have been increasingly conducted to estimate percutaneous absorption of chemicals. The following experimental design is commonly used: A piece of excised human skin is attached to a diffusion apparatus that has a top chamber for the test compound, an O-ring to hold the skin in place, and a bottom chamber to collect samples for analysis. The flow of a chemical across the skin can be calculated with models based on chemical thermodynamics, taking into consideration the octanol/water partition coefficients, the saturated concentration in aqueous solution, and the molecular mass of the test compound [154]. However, for routine applications, the method has not been sufficiently evaluated.

Table 26. Evaluation of skin reactions according to the Draize scoring system

Erythema	Score
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness)	4
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (area raised ca. 1 mm)	3
Severe edema (raised more than 1 mm and extended beyond area of exposure)	4

Furthermore, an increasing number of toxicokinetic models for estimating the extent of percutaneous absorption of chemicals has appeared in the literature. Among them, a physiologically based toxicokinetic model was recently developed to describe the percutaneous absorption of volatile and lipid-oluble organic contaminants in dilute aqueous solution [155]. This toxicokinetic model considers both physiological parameters such as volumes of body compartments and blood flow rate, as well as the properties of the test compound. Presently, these models do not play a role in regular toxicity testing and are therefore not discussed in depth here.

More recently extensive progress has been also made in developing in vitro systems for evaluating the dermal irritation potential of chemicals. An overview of the systems that have been evaluated so far for a range of compounds by comparison of their predictive accuracy with animal test results is presented in Table 27 (for a review see [156]).

Table 27. In vitro test systems for detection of dermal irritation potential

System	End point
Mouse skin organ culture	leakage of LDH* and GOT**
Human epidermal keratinocytes	release of labeled arachidonic acid, cytotoxicity
Cultured BHK21/C13 cells	growth inhibition, cell detachment
SKINTEX – protein mixture	protein coagulation

* LDH = Lactate dehydrogenase.
** GOT = Glutamic acid oxalacetic transaminase.

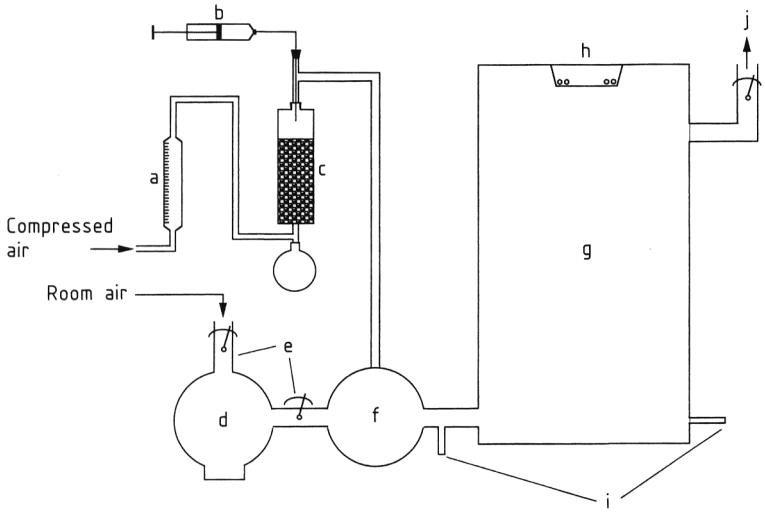


Figure 60. Example of an open inhalation chamber for exposure to volatile liquids
a) Meter; b) Syringe coupled to infuser; c) Heated beads of silica or glass; d) Compressor; e) Metering valves; f) Mixing chamber; g) Exposure chamber; h) Ventilation; i) Sampling valves for determination of atmospheric concentration; j) Exhaust

When evaluating these studies, attention should be given to the occurrence of persisting irritating effects, even those which do not lead to classification. Effects such as erythema, oedema, fissuring, scaling, desquamation, hyperplasia, and opacity which are not reversible within the test period may indicate that a substance will cause persistent damage to the human skin and eye.

4.2.3. Testing for Acute Toxicity by Inhalation

Studying the toxicity of a chemical by inhalation exposure requires a considerable technological input. Therefore, inhalation exposure is usually not tested if this absorption pathway is not expected to occur because the test chemical is not volatile or the physicochemical properties of solids do not allow the generation of respirable particles. Particles with diameters greater than 100 μm are unlikely to be inhaled, because they settle too rapidly. Particles with diameters of 10–50 μm are likely retained in the nose and the upper parts of the respiratory tract, while particles with diameters of less than 7 μm can reach the alveoli of the human lung. When performing toxicity studies with inhalation exposure, the differences in respiratory physiology between humans and the small laboratory rodents must be considered. In contrast to humans, the rat is an obligate nose breather with a complex nasal turbinate structure which filters many small particles. Therefore, the upper size limit for particles reaching the alveolar region in rats is in the range of 3–4 μm in diameter.

The duration of exposure in acute inhalation studies is usually 4–6 h, and they may be performed either as whole-body or head(nose)-only procedures in specific exposure chambers. A number of important considerations should be taken into account in planning and evaluating inhalation studies. The frequently made assumption that on the basis of their physical form, gases and vapors will be absorbed uniformly throughout the respiratory tract is incorrect. Many gases or vapors, such as ammonia, formaldehyde, and sulfur dioxide have high solubility in water and are rapidly absorbed by the humid epithelial surface of the upper respiratory tract. Therefore, toxic effects observed after inhalation of this type of chemical will generally be confined to these regions, especially to the nasal passages. In contrast, chemicals with low solubility in water such as nitrogen dioxide, phosgene, and ozone will penetrate readily to the low pulmonary regions, even at relatively low concentrations in the respiratory air. In mixed atmospheres con-

taining both a gas or vapor and particulates, the vapor or gas may be absorbed on the particulate fraction, so that the deposition pattern of the vapor or gas is governed by the size of the particulate fraction and not by the water solubility of the vapor or gas.

The inhalation exposure of experimental animals may be performed in a dynamic or static mode. In dynamic systems, the test atmosphere is continuously renewed, ensuring atmospheric stability and constant concentrations of the chemical in the gas phase. This mode of inhalation exposure is more complicated and requires larger amounts of the test chemical and suitable systems to ensure complete mixing of the continuously applied test compounds with the stream of air flowing through the exposure system (Fig. 60).

Static systems are sealed, and the atmosphere is circulated. The concentration of the chemical in the gas phase decreases during exposure due to its uptake and biotransformation by the experimental animals. Static systems are used predominantly in acute toxicity studies and in research laboratories because they are relatively inexpensive and consume only a small amount of the chemical in comparison to the amounts need to generate a dynamic exposure. In analogy to the LD_{50} values obtained in the acute oral studies the mean lethal air concentrations LC_{50} are assessed in acute inhalation studies, usually for an exposure time of 4 h (Fig. 61).

4.3. Repeated-Dose Toxicity Studies: Subacute, Subchronic and Chronic Studies

Repeated-dose toxicity studies assess the effects resulting from the accumulation of a compound or its toxic effects in the organism and, unlike acute studies, they can also reveal toxic effects that appear after a latency period. A classic example is the delayed neuropathy caused by some organophosphorous insecticides and by cresyl phosphates, which is manifested several weeks after the first administration of the test compound. In contrast to the marked clinical symptoms observed in the course of delayed neuropathy, these compounds hardly cause any acute symptoms immediately after the first administration. Hence, false negative results may be obtained if an assessment were based only acute toxicity testing. The major aims of repeated-dose toxicity studies are the identification of starting points for the extrapolations required in human risk assessment such as NOAEL and benchmark doses, and the identification of critical end points to be carried over into the risk assessment process.

Testing for subacute toxicity is usually performed over a time period of 2–4 weeks at three dose levels as an aid in selecting the dose levels for the subchronic studies. Studies to determine subchronic effects are usually performed in rats and dogs over 10% of the animals life-span (3

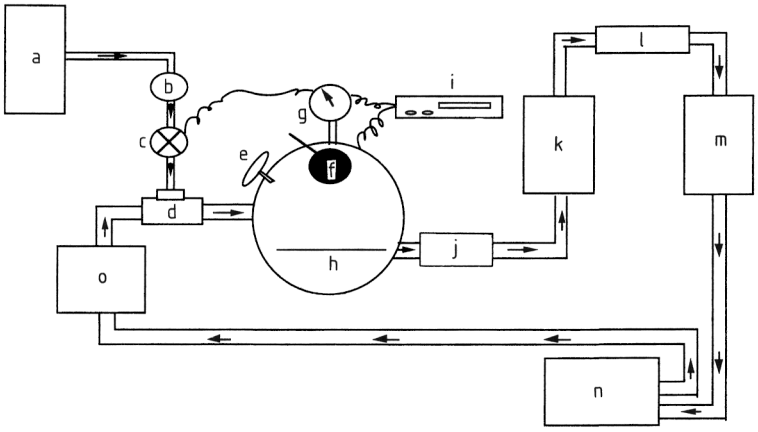


Figure 61. Closed exposure system
a) Oxygen cylinder; b) Metering valve; c) Solenoid valve; d) Mixing chamber; e) Thermometer; f) Oxygen sensor; g) Pressure gauge; h) Exposure chamber; i) Oxygen monitor; j) Injection port; k) Condenser; l) Flow meter; m) Carbon dioxide absorber; n) Gas chromatograph; o) Pump

months in rats, 12 months in dogs). At the start of the study, rodents should be 6–8 weeks old and dogs 4–6 months old. The animal numbers enrolled are between 10 and20 rats and 6 and 8 dogs per sex per dose group. Ideally, the lowest dose of the chemical should not induce toxic effects, the intermediate dose should induce slight toxicity, and the high dose should induce clear signs of toxicity without causing death in more than 10% of the animals.

In both subchronic and chronic toxicity studies (see below), the test compound is often incorporated into the diet or added to the drinking water. Food consumption varies from weanling to maturity, with younger animals consuming more food on a bodyweight basis. Therefore, it is necessary to predict the changes in body weight and food consumption on a weekly basis and adjust the concentration of the test compound in the diet in order to ensure constant dosing throughout the study. Compounds not stable in diet or water or not accepted by the animals may also be applied by gavage. Application by gavage directly into the stomach ensures constant dosing, but gavage studies require skilled personnel, and gavage-related trauma may reduce survival in all groups. Oral application with the feed is usually performed on a 7 days per week basis, while a 5 days per week scheme is frequently used for gavage administration, skin application, and inhalation studies.

Studies on the chronic toxicity of chemicals are usually performed for at least six months in rodents and 12 months in dogs, the chronic toxicity studies can be combined with a carcinogenicity study. Dose levels are usually selected on the basis of the results of studies on acute and subacute toxicity. The highest dose applied should be toxic, i.e., suppress body weight up to 10% (maximum tolerated dose, MTD). The two other dose-levels are usually 1/4 and 1/8 of the MTD. Xenobiotics showing no adverse effects in the short-term studies are usually tested at doses which are 100–200 times higher than the expected human exposure.

In both subchronic and chronic studies, cage-side examination and clinical chemistry are performed routinely during the test period. After termination of the study, much emphasis is placed on the histopathological evaluation of treatment-induced adverse effects.

Cage-side observations during the study

1) Body weight, food and water consumption
2) Skin and fur, eyes, mucous membranes
3) Respiration and blood circulation
4) Motor activity and behavioral pattern

Clinical chemistry during the study

1) *Blood:* erythrocyte, leukocyte, and differential leukocyte counts; hemoglobin concentration; hematocrit, platelet, and reticulocyte counts; electrolytes; inorganic phosphorus and alkaline phosphatase; glucose, protein, albumin, creatinine, urea, lipids, enzymes
2) *Urine:* volume and coloration/turbidity, osmolality and pH, glucose and protein, urine enzymes and cytology

Toxicologic pathology after termination of the study and in animals dying during the study

1) Organ weights and macroscopic evaluation
2) Histopathological examination of brain, liver, kidney, spleen, testes, and every organ with macroscopic changes

4.4. Ophtalmic Toxicity

The majority of injuries to the eyes by direct contact with a chemical occur with substances which are handled in an uncontrolled manner, e.g,. by children in the home; and this type of injury is easily prevented at the occupational setting by using simple protective procedures. However, many irritant gases and vapors may also produce ophtalmic toxicity, and these toxic effects are of practical importance in occupational medicine.

The conventional in vivo eye irritation test in the rabbit was formalized by Draize some 50 years ago and still remains the only fully validated method to assess ophtalmic toxicity. Increasing criticism primarily based on the discomfort and the deliberate injury caused to the animals led to the development of several in vivo and in vitro alternative methods, which will be described at the end of this chapter.

In the conventional in vivo test the chemical (0.1 ml of liquid or 100 mg of solid chemical) is instilled into one eye of each of the test rabbits, the contralateral eye serving as control. Eyes are

then examined periodically (usually after 1, 24, 48, 72 h and 7 dd) and the ocular lesions are scored essentially according to Draize et al.:

Cornea

A)	Opacity	
	No opacity	0
	Scattered or diffuse area, iris details clearly visible	1
	Easily discernible translucent areas, iris details slightly obscured	2
	Opalescent areas, iris details not visible, pupil size barely discernible	3
	Opaque, iris invisible	4
B)	Area of cornea involved	
	One-quarter or less, but not zero	1
	Greater than one-quarter, but less than half	2
	Greater than half, but less than three-quarters	3
	Greater than three-quarters, up to whole area	4

Corneal score = (A) × (B) × 5 (maximum total score = 80)

Iris

A)	Normal	0
	Folds above normal, congestion, swelling, circumcorneal injection (any or all), iris still reacting to light	1
	No reaction to light, hemorrhage, gross destruction	2

Iris score = (A) × 5 (maximum total score = 10)

Conjunctivae

A)	Vessels normal	0
	Vessels definitely injected, above normal	1
	Diffuse, deep crimson red, individual vessels not readily discernible	2
	Diffuse beefy red	3
B)	No chemosis (swelling)	0
	Any swelling above normal (includes nictitating membrane)	2
	Obvious swelling with partial eversion of lids	2
	Swelling with lids about half closed	3
	Swelling with lids about half to completely closed	4
C)	No discharge	0
	Any amount of discharge different from normal	1
	Discharge with moistening of lids and hairs adjacent to lids	2
	Discharge with considerable moistening around eyes	3

Conjunctival score = [(A) + (B) + (C)] × 2 (maximum total score = 20)

Total maximum score (cornea + iris + conjunctiva) = 110

There is increasing evidence that a volume of 0.01 ml of liquid xenobiotics is as sensitive as the conventionally used 0.1 ml and is probably more appropiate for comparison with human exposure situations. Ocular toxicity testing for exposure to gases, vapors, and aerosols is carried out in appropiate exposure chambers.

A number of in vivo and in vitro alternatives to the conventional eye irritation test have been suggested. The in vivo alternatives aim at reducing discomfort of the animals by employing lower doses of the test material and increasing the sensitivity of the test by using noninvasive objective measurements. Among them, the assessment of corneal thickness and of the intravascular pressure seem to be sensitive parameters to identify mild to moderately irritant chemicals. In spite of the large number of suggested in vitro tests, currently no single in vitro test has proved effective in predicting the eye irritation. Therefore, in vitro tests can not replace the rabbit eye test yet. However, in vitro assays are useful as screens for product development to reduce the number of tests performed in animals later. A number of these tests are presented in [157].

4.5. Sensitization Testing

Chemicals that have the potential to elicit allergic reactions are continously introduced into the human environment. Therefore, allergic reactions of the skin are becoming an increasingly important problem, especially in the workplace. Allergic contact dermatitis is one of the most common occupational diseases and may become debilitating unless the causative agent is identified and exposure stopped. While irritant dermatitis is generally produced by direct interaction of the chemical with skin constituents, allergic dermatitis is the result of a systemic immune reaction which in turn induces effects in the skin. An important characteristic of allergic reactions that must be taken into account when testing for allergenic potential, is that allergic responses usually have a biphasic course. The induction period between initial contact with the causative agent and the development of skin sensitivity may be as short as two days for strong sensitizers such as poison ivy extract, or may require several years for a weak sensitizer such as chromate; for most of the chemical compounds with allergenic potential the induction period usually takes from 10 to 21 d. After this initial development of sensitivity to a certain allergenic chemical, the time between reexposure to this agent and the occurrence of clinical allergic symptoms is generally between 12 and 48 h; in animal testing, this period is called the challenge phase.

Table 28. Guinea pig sensitization tests

Test	Induction: route/number of applications	Challenge: route/number of applications
Draize	intradermal/10	intradermal/1
Open epicutaneous	epidermal open/20	epidermal open/1
Buehler	epidermal occlusive/3	epidermal occlusive/1
FCA*	intradermal in FCA*/3	epidermal open/1
Split adjuvants	epidermal occlusive/4 + FCA* intradermal/1	epidermal occlusive/1
Optimization	intradermal + FCA*/10	intradermal/1 epidermal occlusive/1
Maximization	intradermal + FCA*/1 epidermal occlusive/1	epidermal occlusive/1

* FCA = Freund's complete adjuvant.

The general objectives are to determine whether there are indications from human experience of skin allergy or respiratory hypersensitivity following exposure to the agent and whether the agent has skin sensitization potential based on tests in animals. There are two methods currently described in EU Annex V and OECD guidelines for skin sensitization in animals: the guinea pig maximisation test (GPMT) and the Buehler test. The GPMT is an adjuvant-type test in which the allergic state (sensitization) is potentiated by the use of Freund's Complete Adjuvant (FCA). The Buehler test is a non-adjuvant method involving topical application for the induction phase rather than the intradermal injections used in the GPMT (Table 28; for reviews see references [158, 159]. Although they differ by route and frequency of treatment, they all utilize the guinea pig as test species. In general, for the induction phase the chemical is administered to the shaved skin intradermally, epicutaneously, or by both routes several times over a period of two to four weeks. Freund's complete adjuvant (FCA, a mixture of heat-killed *Mycobacterium tuberculosis*, paraffin oil, and mannide monooleate) is often included to increase the immunological response. During the challenge phase, a nonirritating concentration of the chemical is applied. The concentration of the test chemical and the application route (epi- or intradermal) are often different between the two phases. Sensitization is assessed by examining the skin reactions (edema, erythema) following the challenge phase and comparing them with any skin reactions observed immediately after the induction phase; the latter reactions are considered to result from direct irritating (toxic) properties of the test chemical. Hence, the difference between the symptoms observed after the induction and after the challenge phase is attributed to the allergenic effects of the chemical.

The guinea pig maximization test is the most widely used and is considered to be very sensitive. The first part of the induction phase includes simultaneous injection of FCA alone, the test compound in saline, and the test compound in FCA into three different areas in close proximity to each other. The second part of the induction phase 7 d later employs epicutaneous application of the chemical on a filter paper, which is occluded and left in place for 48 h. The challenge phase is conducted epicutaneously for 24 h, two weeks after the induction phase. The maximization test is very sensitive and may produce false positive results. The original procedure (injection of the test compound) does not allow testing of final product formulations. Therefore, a modified procedure has been developed. In the first week, the FCA is injected four times and the test product formulation is administered epidermally, as in the second induction week.

Both the GPMT and the Buehler test have demonstrated the ability to detect chemicals with moderate to strong sensitization potential, as well as those with relatively weak sensitization potential. These guinea pig methods provide information on skin responses, which are evaluated for each animal after several applications of the substance, and on the percentage of animals sensitized.

The murine local lymph node assay (LLNA) is another accepted method for measuring skin sensitization potential. It has been validated internationally and has been shown to have clear animal welfare and scientific advantages compared with guinea pig tests. In June 2001, the OECD recommended that the LLNA should be adopted as a stand-alone test as an addition to the existing guinea pig test methods.

Respiratory hypersensitivity is a term that is used to describe asthma and other related respiratory conditions, irrespective of the mechanism by which they are caused. When directly considering human data in this document, the clinical diagnostic terms asthma, rhinitis, and alveolitis have been retained.

There are currently no internationally recognised test methods to predict the ability of chem-

icals to cause respiratory hypersensitivity. Potentially useful test methods based on allergic mechanisms are the subject of research and development. However, there are currently no test methods under development which are designed specifically to identify chemicals that cause respiratory hypersensitivity by nonimmunological mechanisms.

4.6. Phototoxicity and Photosensitization Testing

The biologically active spectrum of light can be divided into UV (220–400 nm) and visible light (400–760 nm). The UV spectrum is further divided into UVA (315–400 nm), UVB (280–315 nm), and UVC (220–280 nm); the last-named is absorbed in the stratosphere and does not reach the surface of the earth. The primary source of toxic effects on the skin is UVB, although UVA may also play a critical role in some reactions.

Xenobiotics localized within the skin may be activated by UVB and induce phototoxicity and/or photosensitization (photoallergy). Photoallergy is similar, both mechanistically and clinically, to allergic contact dermatitis, the only difference being that the chemical must react with light to become allergenic. Photoallergic reactions are not necessarily dose-dependent and show great variability between individuals. In analogy, phototoxicity may be compared with irritant dermatitis. Many phototoxic reactions may be caused by the formation of free radicals followed by lipid peroxidation and localized inflammation. In addition to phototoxicity and photoallergy, light-induced activation of chemicals may cause depigmentation, induction of an endogenous photosensitizer or of a disease characterized by photosensitization such as lupus erythematodes or pellagra. However, these reactions are comparatively rare. The tests to identify photoallergenic and phototoxic chemicals are performed in analogy to the tests for skin sensitization and irritation, respectively.

Photoallergenic potential is evaluated by repeated application of the test compound on the skin of guinea pigs and exposure of the treated area with UV light after every application; the UV treatment should cause a very slight erythema. Several days after this induction phase, the challenge phase is conducted by treatment with a low dose of the test compound together with UV light. Phototoxic reactions can usually by observed after the first exposure to the test compound together with UV light; in addition to guinea pigs, mice and rabbits are also utilized in tests for phototoxicity.

4.7. Reproductive and Developmental Toxicity Tests

The general objectives of reproductive and developmental toxicity testing are to establish whether exposure to the chemical may be associated with adverse effects on reproductive function or capacity, and whether administration of the substance to males and/or females prior to conception and during pregnancy and lactation causes adverse effects on reproductive function or capacity. Another focus of these studies are induction of nonheritable adverse effects in the progeny and whether the pregnant female is potentially more susceptible to general toxicity.

Reproductive and developmental toxicity is a very broad term including any adverse effect on any of the following aspects:

1) Male or female sexual structure and function (fertility)
2) Development of the new organism through the period of major organ formation, organogenesis (embryotoxicity and teratogenicity)
3) Development of the new organism during the peri- and postnatal periods

The field of reproductive toxicology has become increasingly important with the recognition that viral and bacterial infections and xenobiotic chemicals can produce severe and irreversible defects in the offspring. The first reports on malformations due to rubella virus infections, ionizing radiation, hormones, dietary deficiencies, and chemicals appeared in the 1930s and 1940s, but the potential impact of reproductive toxicity on public health was only recognized more than two decades later. In 1960, a large increase in newborns with specific limb malformations, which are rarely seen otherwise, was recorded in Germany and in other parts of the world. One year later, the sedative/hypnotic drug thalidomide was recognized as the causative agent. The thalidomide epidemic resulted in

10 000 malformed children and subsided after the drug was withdrawn from the market at the end of 1961. One important consequence of the thalidomide disaster was the introduction of requirements for the testing of potential new drugs for reproductive toxicity. All test batteries required include detailed tests for reproductive toxicity to prevent a repetition of the thalidomide disaster.

The first specific reproductive toxicity test to be conducted is usually the two-generation study. which should be initiated after the rat 90-d subchronic repeated-exposure study, since the results obtained may provide information necessary for selecting dose levels for the two-generation study. Additionally, repeated-exposure studies that can provide information relevant to reproductive toxicity should be used in the design of the two-generation study. For example, the observation of neurological effects may indicate the need to evaluate developmental neurotoxicity.

The first developmental toxicity study is normally performed after completion of the two-generation study. The design of the developmental toxicity study should use all information derived from the repeated-exposure and two-generation studies, in particular dose–response relationships and information on maternal toxicity. The preferred species for the two-generation study is the rat; the necessity of a developmental toxicity study in the rabbit is dependent on the outcome of the first study.

4.7.1. Fertility and General Reproductive Performance

Segment I experiments are usually conducted in rats (20 animals of each sex per dose) with three doses of the test chemical, most often administered with diet. The treatment must not cause general systemic toxicity in the parental organism; therefore, in dose selection the dose levels are chosen according to observations in studies of subacute and subchronic toxicity, which are usually performed before testing for reproductive toxicity. Young adult male rats are treated for 60–80 d prior to mating to cover a whole period of spermatogenesis. Female rats are pretreated for 14 d to cover three estrous cycles. Treatment of both sexes is continued during the mating period and that of females throughout pregnancy. Half of the females are sacrificed just before term, and the numbers of resorbed and dead fetuses as well as structural abnormalities in the developed fetuses are assessed. In the United States, pregnancy is interrupted midterm in half of the females. Treatment of the remaining females is continued through parturition and lactation until weaning of the newborns, usually 21 d after birth. The young animals (F_1 generation) are reared without receiving the test compound until sexual maturity, when their fertility is assessed. During the rearing period, the development of the young animals is monitored with cage-side carefully clinical observations. If an adverse effect on fertility, pregnancy, or development of the offspring is observed, it is necessary to evaluate whether the effect is due to toxicity to the male or female reproductive system or both. This information can be obtained by separate mating of treated males with untreated females and vice versa.

Furthermore, evaluation of toxic effects on the male and female reproductive systems with specific test systems may be required. The effect of the test compound on male reproductive performance may be evaluated by monitoring mating behavior (e.g., frequency of copulation). Structural and functional impairment of the male reproductive organs is assessed by conducting gross pathology and histology of the testes and sperm analysis (viability, motility, and morphology). Histological examination of the ovaries plays an important role in assessment of toxic effects on the female reproductive system.

4.7.2. Embryotoxicity and Teratogenicity

Segment II studies assess adverse effects during the period of organogenesis. Xenobiotics interfering with the developing organism during this extremely sensitive period may cause severe and irreversible structural malformations. These studies are carried out in two species, usually rats (20 per dose) and rabbits (10 per dose); in most cases, two dose levels and an untreated control group are included. Pregnant animals are tested during the period of organogenesis: days 6 to 15 for rats and 6 to 18 for rabbits. The fetuses are delivered by cesarean section one day prior to the estimated time of delivery: day 21

for rats and day 31 for rabbits. The main reason for avoiding natural delivery is to prevent loss of deformed or dead fetuses by cannibalism, which happens in rodents and rabbits. The uterus of the maternal animal is excised, weighed, and examined for implantation sites and resorbed fetuses. The pups are weighed, and one-half of each litter is usually examined for skeletal defects and the remaining one-half for soft-tissue defects.

4.7.3. Peri- and Postnatal Toxicity

For segment III studies, treatment of pregnant rats with three dose levels (10–12 animals per dose) begins on day 16 of gestation and is carried on through delivery and lactation, until weaning of the offsprings, normally on day 21 postpartum. Treatment during parturition and lactation is also performed in segment I studies; however, segment III studies may offer additional information since higher doses can be used than in segment I. The peri- and postnatal segment evaluates effects on birth weight and survival as well as development of the offspring in the postnatal period. However, extrapolation of results of segment III studies from the rat to the human situation should be performed with care and consideration of the specific circumstances in each species. In contrast to the human situation, where in addition to the mother the social environment takes care of the newborns, young rats depend completely on the functional integrity of the maternal organism. Furthermore, the organism of newborn rats is significantly less mature by the time of delivery than that of newborn humans. Hence, treatments that affect the maternal organism simply by causing sedation or fatigue may significantly impair the development of newborn rats in the first 21 days of life. Such effects should not be automatically interpreted as relevant for postnatal toxicity in humans.

4.7.4. Multigeneration Studies

Multigeneration studies assess the cumulative effects of continuous application of the test chemical on reproduction and development during two or three generations. This application mode is relevant for long-term exposure to chemicals in the environment, such as pesticide residues in food or contamination of drinking water with agricultural chemicals or nonbiodegradable solvents.

The two-generation study is a general test which allows evaluation of the effects of the test substance on the complete reproductive cycle including libido, fertility, development of the conceptus, parturition, postnatal effects in both dams (lactation) and offspring, and the reproductive capacity of the offspring. The two-generation study is preferable to the one-generation study because the latter has some limitation regarding assessment of post-weaning development, maturation, and reproductive capacity of the offspring. Thus, some adverse effects such as oestrogenic- or antiandrogenic-mediated alterations in testicular development may not be detected. The two-generation study provides a more extensive evaluation of the effects on reproduction because the exposure regime covers the entire reproductive cycle, permitting an evaluation of the reproductive capabilities of offspring that have been exposed from conception to sexual maturity. The prenatal developmental toxicity study only provides a focused evaluation of the potential effects on prenatal development.

Three dose levels are usually given to groups of 25 female and 25 male rats shortly after weaning at days 30 to 40 of age. In the multigeneration study, these rats are referred to as the F_0 generation. The F_0 generation is treated throughout breeding, which occurs at about 140 d of age, and the female animals also during pregnancy and lactation. Hence, the offspring (F_1 generation) has been exposed to the test compound in utero, via the maternal milk, and thereafter in the diet. In many protocols, the F_1 generation is standardized to include certain numbers of animals, e.g., eight animals per litter. In analogy to the F_0 generation, the F_1 generation is bred at about 140 d of age to produce the F_2 generation. In some of the parents (F_0 and F_1 generations), gross necropsy and histopathology is conducted with greatest emphasis on the reproductive organs. In addition, necropsy and histopathology are carried out in all animals dying during the study.

The percentage of F_0 and F_1 females that become pregnant, the number of pregnancies carried to full term, the litter size, and number of re-

sorptions, stillborns, and live births are recorded. Viability counts and pup weights are recorded at birth, and at days 4, 7, 14, 21 and 28 of age. With these data, the following parameters are calculated for assessment of the long-term reproductive toxicity of the test compound:

$$\text{Fertility index (\%)} = \frac{\text{Number of pregnancies}}{\text{Number of matings}} \times 100$$

$$\text{Gestation index (\%)} = \frac{\text{Number of litters}}{\text{Number of bred females}} \times 100$$

$$\text{Birth index (\%)} = \frac{\text{Number of pregnancies resulting in live off spring}}{\text{Number of pregnancies}} \times 100$$

$$\text{Viability index (\%)} = \frac{\text{Number of animals alive at day 4 after birth}}{\text{Number of new borns}} \times 100$$

$$\text{Lactation index (\%)} = \frac{\text{Number of animals alive at day 28 after birth}}{\text{Number of animals alive at day 4 after birth}} \times 100$$

4.7.5. The Role of Maternal Toxicity in Teratogenesis

If an agent with selective developmental toxicity is administered throughout the organogenesis period (days 6 to 15 in the rat), identifying the most sensitive target organs becomes difficult. In addition, teratogenic effects may be masked by embryolethality with repeated dosing during the organogenesis period. Developmental toxicity in the form of increased resorption and decreased fetal body weight is generally accepted to occur at maternally toxic dose levels. The role of maternal toxicity in causing congenital malformations, however, is not clear. Doses causing maternal toxicity, as indicated by reduced maternal body weight, clinical signs of toxicity, or death, commonly cause reduction in fetal body weight, increased resorption, and rarely, fetal deaths. Three patterns of association between maternal toxicity and malformations can be observed: (1) for some compounds, maternal toxicity is not associated with malformations; (2) for others, maternal toxicity is associated with a diverse pattern of malformations, often including cleft palate; and (3) the maternal toxicity of still others is associated with a characteristic pattern of malformations.

Compounds in the second category are the most difficult to classify in terms of teratogenic potential. Cleft palate is the principal malformation resulting from food and water deprivation during pregnancy in mice; however, cleft palate is also a malformation specifically induced in mice by a number of teratogens, most notably the glucocorticoids, without apparent maternal toxicity. Complete determinations of food and water consumption, maternal body weights, and impairment of the maternal organism are necessary to distinguish between cleft palate caused by the teratogenic effect of a chemical on the embryo and that resulting from systemic maternal toxicity, which secondarily affects embryonic development. The association of maternal toxicity with major malformations, such as exencephaly and open eyes, is not generally accepted, although most investigations agree that maternal toxicity can cause minor structural abnormalities such as variants in the ribs.

4.7.6. In Vitro Tests for Developmental Toxicity

Models to elucidate the mechanism of embryogenesis have been under development for several decades, and therefore developmental toxicology is a fields in which alternative methods to animal experimentation are available. However, because of the complicated, multistep nature of the development of a new life, none of the in vitro systems presently available can replace the animal tests. In vitro tests rather serve for screening purposes, i.e., to preclude the extensive traditional whole-animal test protocol for compounds with marked toxicity on reproduction and development. The existing alternative test systems fall into six groups: lower organisms, cell-culture systems, organ-culture systems, whole-embryo cultures, embryos, and others (Table 29). Since none of the in vitro methods is sufficiently validated for a set of compounds for which the effects on humans or animals are known and the field is much too extensive to be comprehensively reviewed here, the reader is referred to two comprehensive reviews of this field [160].

Table 29. In vitro test systems for developmental toxicity

Group	Test system/organisms	End points monitored
Lower organisms and small animals	sea urchins	growth
	drosophila	
	trout, medaka (fish species)	
	plania	
	brine shrimp	
	animal virus	
Cell culture	pregnant mouse and chick lens epithelial cell	protein synthesis
	avian neural crest	cell differentiation
	neuroblastoma	cell differentiation
Organ culture	frog limb	regeneration
	mouse embryo limb bud	morphological and biochemical differentiation, toxicity
	metanephric kidney organ culture from day 11 mouse embryos	morphological and biochemical differentiation
Whole embryo cultures	chick embryo	embryotoxicity, malformations
	frog embryo teratogenesis assay	lethality, no observed-effect-level, development stage, attained growth, motility, pigmentation, gross anatomical malformations
	rat embryo culture (postimplantation embryo)	viability, growth and macromolecular content, gross structural and histological abnormalities

Table 30. Basic procedures of rodent carcinogenicity bioassays

Species	rat (Fischer 344, Sprague – Dawley, Wistar)
	mouse (B6C3F1, CD)
Age of animals at the beginning	4 to 6 weeks (shortly after weaning)
Number of animals	50 per sex per dose for carcinogenicity
	10 – 20 for additional studies during the course of experiment
Dose	at least three doses and vehicle control
	maximum tolerated dose
	intermediate dose
	nontoxic dose
Duration	24 months
Application	gavage, in feed, drinking water, inhalation (only if absolutely necessary)
Toxicologic pathology	all animals: gross necropsy
	weight of all important organs
	histopathology of all tissues (ca. 40) and all tumors and preneoplastic lesions by two independent pathologists

4.8. Bioassays to Determine the Carcinogenicity of Chemicals in Rodents

Despite the many available short-term in vivo and in vitro tests to determine the genotoxic and carcinogenic potential of chemicals and the vast amount of literature on the subject, the lifelong carcinogenicity bioassay remains the main instrument for reliable evaluation of the carcinogenic properties of a xenobiotic. The principal guidelines for performing bioassays were established some 25 a ago by the U.S. National Cancer Institute and have essentially been adopted with slight alterations by all regulatory authorities (Table 30).

A number of factors may interfere with the analysis and interpretation of data from animal carcinogenicity studies. A variety of statistical techniques has been developed to adjust for confounding factors and to estimate confidence intervals and significance of results. Significance tests are used to assess neoplastic response in treated groups as compared to control groups or historical controls (cancer incidence in the identical strain and species observed in control groups for other cancer bioassays under identical housing conditions in the same facility). To estimate the absolute cancer risk posed by a specific chemical, background or spontaneous cancer incidences (induction of neoplasms not related to the administration of the test chemical) must be well defined. In general, high background incidences of cancer such as liver cancer observed in specific strains of mice requires larger number of animals in the treatment groups to detect increases in cancer incidence induced by the administration of the test chemical and to obtain statistically significant results. The demonstration of a dose–response curve for the cancer incidence in groups of animals treated with different doses of the carcinogen will increase the confidence in positive results of an animal cancer bioassay. The same holds for identical results observed with cancer as an endpoint in an independent study.

However, despite the importance of animal cancer bioassays for characterizing chemical carcinogens, this approach has been criticized recently (see also Section 5.2.4.4). Due to the influence of rodent carcinogenicity assay on the development of new chemicals and pharmaceu-

tical drugs, the pros and cons of this type of assay are discussed in depth in the following.

Practically all chemicals identified as human carcinogens produce tumors in the rodent bioassay. Hence, the test has a very good predictive value, and every chemical exerting carcinogenicity in rodents should be handled as a potential human carcinogen. Considering the many new chemicals developed each year, two major disadvantages of the life-long assay are its high cost and long duration. A two-year gavage study in only one species amounts to approximately €10^6 and takes 3–4 years or longer for complete evaluation. For optimized evaluation of carcinogenic properties, it is important to use animal species that are closest to humans with regard to biotransformation and toxicokinetics of the test compounds. However, for practical and financial reasons, only rodents can be used. Long-term carcinogenic bioassays in dogs or primates, for example, require seven to ten years for completion and are much more costly.

The application of the maximum tolerated dose (MTD) in rodent bioassays has been the subject of much controversy [161–167], but the experimental design limitations of in vivo studies make the application of high doses necessary. For example, if a specific dose of a chemical causes a 0.5% increase in human cancer incidence, this would result in several hundred thousands of additional cancer cases in a country such as Germany each year and would thus definitely pose an unacceptable risk. However, the identification of this 0.5% increase in cancer incidence with statistical confidence in the rodent bioassay would require a minimum of 1000 animals, provided the incidence of spontaneous tumors is zero. Therefore, there seems to be general agreement that the use of the MTD, although not an optimum solution, is necessary for risk assessment. According to the U.S. National Cancer Institute, the MTD is defined as "the highest dose that can be predicted not to alter the animals' normal longevity from effects other than carcinogenicity". In practical terms "MTD is the dose which, in the subchronic three-months toxicity study causes not more than a 10% weight decrement as compared to the control groups and does not produce mortality, clinical signs of toxicity, or pathological lesions other than those which may be related to a neoplastic response that would be predicted to shorten an animal's life span". As stated above, the MTD is determined in the preliminary three-month studies on subchronic toxicity, where it fulfills the above requirements. Ideally, this is exactly what should also happen in the 24-month carcinogenicity study. However, due to cumulation of toxic effects and/or alterations in toxicokinetics of the xenobiotics during the study, for example, induction of toxification or detoxification pathways by application of the xenobiotic in high doses, the MTD dose group often shows reduced survival rates in the life-long bioassay. This may invalidate the study, that is, make it inadequate for evaluation of the carcinogenic potential. Indeed, this is not a rare event in carcinogenicity studies. The opposite effect may also occur: due to toxicokinetic differences between the three- and 24-month studies, the MTD chosen may turn out too low in the long-term bioassay. In spite of all these problems and because of the absence of a satisfactory alternative solution, the use of the MTD is currently the only method to compensate for the fact that in relation to the human population exposed to potential carcinogens, the numbers of rodents used in the carcinogenicity bioassay are extremely low. The legitimate argument against the MTD is that any chemical given at a sufficiently high dose level will induce adverse effects. This understanding, which is beyond dispute in toxicology, has been tentatively generalized by several scientists in recent years by the notion that carcinogenic effects obtained at the MTD may exclusively result f rom target-organ toxicity, and the increased cell proliferation may contribute to tumor formation by increasing the rate of spontaneous mutations, since DNA replication does not take place with 100% fidelity. Furthermore, during increased cell turnover, the time available to repair DNA damage is reduced, so that an increased number of damaged DNA sites may be converted to heritable mutations. Although this may be the mechanism underlying the carcinogenic effects of some nongenotoxic chemicals, it can not be generalized to every tumor observed at the MTD. Toxicity and cell proliferation do not necessarily result in tumor formation. Table 31 summarizes the important differences between rodent bioassays and human exposure to carcinogens.

Table 31. Some important differences between carcinogenicity tests in rodents and human exposure to potential carcinogens

Rodent carcinogenicity test	Human exposure
High doses	(usually) low doses
Continuous exposure	(often) infrequent or not regular exposure
Single compound, no interactions	simultaneous exposure to several carcinogenic chemicals, interactions probable
Homogeneous population	heterogeneous population

In addition to these problems, which are inherent in the bioassay procedure, the evaluation of the toxicological pathology has repeatedly become an issue of debate, since differences in evaluations between pathologists are frequent. This does not necessarily indicate incompetence of one of the pathologists. The different evaluations may be the result of difference in terminology. Also, sometimes evaluations are conducted years apart, and in the interim period understanding of the pathogenesis of lesions may have changed. Thus, even the same pathologist may not come to the same conclusion when reevaluating tissue slices several years after the first examination.

Due to the uncertainties of rodent bioassays and the extremely high costs and personnel requirements, a multitude of short-term tests has been developed in recent years. These tests aim to predict the carcinogenic potential of chemicals. Most of these in vitro tests are based on damage to the genetic material (genotoxicity) by the chemical or its metabolites. Genotoxicity is without doubt the field in toxicology with the best established and validated in vivo and in vitro short-term tests.

4.9. *In Vitro* and *In Vivo* Short-term Tests for Genotoxicity

Genetic toxicology a comparatively new field of research that has rapidly grown since the 1960s, deals with mutagenicity and genotoxicity.

Mutagenicity is the induction of permanent transmissible changes in the genetic material of cells or organisms. Changes may involve a single gene or gene segment, a block of genes, or whole chromosomes. Effects on whole chromosomes may be structural and/or numerical.

Genotoxicity is a broader term and refers to potentially harmful effects on genetic material which may not be associated with mutagenicity. Thus, tests for genotoxicity include systems which give an indication of damage to DNA (no direct evidence of mutation). End points determined here are unscheduled DNA synthesis (UDS), sister-chromatid exchange (SCE), DNA strand breaks, formation of DNA adducts and mitotic recombination.

Evidence has increasingly accumulated that many carcinogens are also mutagenic, and a large number of short-term in vitro and in vivo tests were developed as predictive tools. Most of these tests are well validated and aim to assess

Table 32. OECD guidelines on genetic toxicology testing and guidance on the selection and application of assays

No.	Title	Original adoption	Updated
471	Bacterial Reverse Mutation Test	26 May 1983	21 July 1997
472	Genetic Toxicology: *Escherichia coli*, Reverse Assay	26 May 1983	21 July 1997*
473	In Vitro Mammalian Chromosome Aberration Test	26 May 1983	21 July 1997
474	Mammalian Erythrocyte Micronucleus Test	26 May 1983	21 July 1997
475	Mammalian Bone Marrow Chromosome Aberration Test	4 April 1984	21 July 1997
476	In Vitro Mammalian Cell Gene Mutation Test	4 April 1984	21 July 1997
477	Genetic Toxicology: Sex-Linked Recessive Lethal Test in *Drosophilia melanogaster*	4 April 1984	
478	Genetic Toxicology: Rodent dominant Lethal Test	4 April 1984	
479	Genetic Toxicology: In Vitro Sister Chromatid Exchange assay in Mammalian Cells	23 Oct. 1986	
480	Genetic Toxicology: *Saccharomyces cerevisiae*, Gene Mutation Assay	23 Oct. 1986	
481	Genetic Toxicology: *Saccharomyces cerevisiae*, Mitotic Recombination Assay	23 Oct. 1986	
482	Genetic Toxicology: DNA Damage and Repair, Unscheduled DNA Synthesis in Mammalian Cells In Vitro	23 Oct. 1986	
483	Mammalian Spermatogonial Chromosome Aberration Test	23 Oct. 1986	21 July 1997
484	Genetic Toxicology: Mouse Spot Test	23 Oct. 1986	

*Date of deletion (method merged with TG 471).

genotoxic properties of chemicals. Today, the majority of potential carcinogens are first identified as mutagens or chromosome-damaging agents in short-term tests and subsequently as carcinogens in the rodent carcinogenicity bioassay. The numerous in vivo and in vitro assays can be categorized into two major groups:

1) Short-term tests detecting gene mutations
2) Short-term tests detecting structural and/or numerical chromosomal aberrations

A number of these in vitro test procedures have gained regulatory acceptance for toxicology testing (Table 32).

In vitro tests to detect gene mutations can be categorized into two groups: microbial and mammalian cell assays. An important step in the history of modern genetic toxicology was the development of genetically precisely defined strains of bacteria carrying mutations in particular genes coding for enzymes involved in the biosynthesis of amino acids. Among the numerous tests, the most widely used and best validated is the assay in *Salmonella typhimurium* developed by Ames et al.

4.9.1. Microbial Tests for Mutagenicity

4.9.1.1. The Ames Test for Bacterial Mutagenicity

The most common method to detect mutations in microorganisms is selecting for reversions in strains that have a specific nutritional (i.e., amino acid) requirement differing from wild-type members of the species; the tester strains are "auxotroph" for this particular nutrient. The *Salmonella typhimurium* mutant strains developed by Ames can not synthesize histidine, because each strain carries one of a number of mutations in in the operon (group of genes) coding for histidine biosynthesis [168, 169]. The result of this mutation is that the tester strains can not grow and form colonies in histidine-free medium. The mutation may revert to the wild-type sequence or a functionally equal sequence either spontaneously (a rare event) or by exposure of the tester strains to genotoxic compounds. The revertant colonies are, like the wild-type bacteria, capable of synthesizing histidine and form colonies in histidine-free medium. For the common tester strains, the DNA sequence at the site of the original mutation in the relevant histidine gene has been determined. According to the type of mutation leading to the inability to synthesize histidine, the strains can be categorized into two groups: base-substitution and the frameshift strains. The difference between these two categories can be illustrated with the following sentence, in which each letter represents a DNA base, each word a triplet coding for an amino acid, and the whole sentence a gene coding for a protein (i.e. enzyme of histidine biosynthesis). The correct sentence represents the gene in the wild-type strain.

THE NUN SAW OUR CAT EAT THE RAT	original sentence (wild-type)
Base-pair substitution:	
THE **S**UN SAW OUR CAT EAT THE RAT	missense mutation coding for a wrong amino acid
THE N**S**N SAW OUR CAT EAT THE RAT	nonsense mutation resulting in interruption of gene transcription
Frameshift mutation:	
THE NUN SA**S** WOU RCA TEA TTH ERA T	+ 1-frameshift mutation

This example illustrates that a base substitution can be reverted by another base substitution and in analogy a frameshift mutation can be reverted by another frameshift mutation. Hence, the Ames test not only provides information on the genotoxic potential of the test compound but also on the nature of the DNA damage.

Genetic Makeup of the *Salmonella typhimurium* Strains Used in the Ames Test. *Histidine mutations of the tester strains*. The base-pair substitution strains can be categorized into two families.

1) The *Salmonella typhimurium* strains TA100 and TA1535 carry the sequence CCC (for leucine) instead of the wild-type sequence CTC (for proline). These missense mutations may be efficiently reverted by mutagens with alkylating properties.
2) The second group of base-pair substitution strains carry a nonsense mutation (TAA instead of CAA). These strains (TA2638, TA100) may detect mutations induced by radicals or oxidizing agents such as hydrogen peroxide and reactive oxygen matabolites.

The commonly used frameshift strains TA98 and TA1538 carry a +1 frameshift mutation near

Table 33. Genetic makeup of commonly used Salmonella typhimurium tester strains

Strain	Histidine mutation		Additional genetic alteration
	Location	Route	
TA100	his G46	CCC instead of CTC	*uvrB*
		base substitution results in proline instead of leucine	*rfa*
			*pkM*101
TA1535	his G46	CCC instead of CTC	*uvrB*
		base substitution results in proline instead of leucine	*rfa*
TA2638	his G428	ATT nonsense mutation	*rfa*
	his G8476	results in interruption of transcription	*pkM*101
TA102	his G428	multiple copies of plasmid pAQ1 carry the revertible nonsense mutation ATT	*rfa*
			*pkM*101
TA98	his D3052	-1-frameshift mutation with a GCGCGCGC sequence	*uvrB*
			rfa
			*pkM*101
TA1538	his D3052	-1-frameshift mutation with a GCGCGCGC sequence	*uvrB*
			rfa

a GCGCGCGC sequence. This strain may be used to detect frameshift mutagens such as polycyclic aromatic hydroxycarbons, certain aromatic amines, and certain aromatic nitro compounds.

In addition to the mutation in one of the genes of histidine biosynthesis, the Ames strains carry additional genetic alterations that increase their sensitivity to detect mutagens (an overview of the genetic make-up of the *Salmonella typhimurium* tester strains is provided in Table 33).

rfa Mutations. The *rfa* mutation results in a defective lipopolysaccharide membrane and thus increases the permeability of the cell wall to bulky hydrophobic chemicals. In addition, strains with defective cell walls are not pathogenic to experimental animals and humans.

uvrB Deletion. Wild-type bacteria possess several effective DNA repair systems, that operate practically error free and can repair DNA damage without allowing mutations to occur. To overcome the problem of DNA repair and thus the decreased sensitivity of the test system, Ames constructed a series of strains with a deletion of the *uvrB* gene, which codes for a subunit of an important enzyme (the cor-endonuclease-I) of the error-free excision repair system. This change increases the sensitivity of the tester strains to mutagens by several orders of magnitude.

The plasmid pkm101. Wild-type *Salmonella typhimurium* strains do not process an "error-prone" DNA repair which is found for example in *Escherichia coli* and some other members of the Enterobacteriaceae. In contrast to the above-mentioned error-free excision repair pathway, the error-prone system operates with low fidelity and introduces new mutations into the genome while repairing a damaged DNA site. To overcome this deficiency and to increase sensitivity to mutagens, the gene for the error-prone repair system has been introduced into some of the *Salmonella typhimurium* tester strains with the plasmid pkm101. This plasmid also carries the genetic information for ampicillin resistance, an important property for monitoring the physiological integrity of the tester strains in the course of the experiments.

The plasmid pAQ1. The *Salmonella typhimurium* strain TA 102 carries the revertible histidine mutation on multiple copies (approximately 30) of the plasmid pAQ 1 and not on the chromosome. Reversion of one of these copies returns the bacteria's capability to synthesize histidine. This increase in sensitivity is partly offset because strain TA 102 has an intact excision repair system (it does not have the *uvrB* deletion). This strain has been constructed because DNA interstrand cross-linking agents such as the quinone mitomycin C or the combination of psoralens und UV light require an intact excision repair system to generate mutations. DNA interstrand cross-links must first be removed from one strand along with a small number of adjacent bases by excision repair. The gap left behind is repaired, and the remaining broken cross-link attached to the other strand is a premutagenic lesion that may give rise to a mutation by error-prone repair.

The Problem of Bioactivation in the Ames Test. The majority of the mutagenic xenobiotics require bioactivation (toxification) to reactive electrophiles to induce DNA damage. The main disadvantage of bacterial mutagenicity assays is that the tester strains do not express many of the enzymes that bioactivate xenobiotics in mammals. For example, cytochrome P450 activity is not detectable with most substrates in *Salmonella typhimurium*. Therefore, there is a need to simulate the biotransformation occurring in the intact animal by supplementing the test system with the enzymes of bioactivation and the necessary cofactors. For this purpose, numerous exogenous metabolic systems have been used in the last decades. They can be grouped into cell-free and cell-based systems, and among the cell-free systems the 9000 *g* supernatant (called S-9 fraction, the S-9-fraction with the cofactors necessary for enzyme activity is called S-9 mix) from rat liver is the most widely used and best validated.

Preparation of S-9 Fraction and S-9 Mix. S-9 fraction is usually prepared from the liver of male adult rats. Preparations from uninduced animals may contain only low activities of important enzymes of bioactivation such as cytochrome P450 1A1. Since these deficiencies could limit the use of the S-9 fraction as exogenous activating systems in bacterial assays, the rats are usually pretreated with enzyme inducers. Among them Aroclor 1254, a mixture of polychlorinated biphenyls, is the most widely used. Pretreatment of the rats with Aroclor 1254 results in induction of a broad range of cytochrome P450 enzymes. For specific purposes, more selective inducers of cytochrome P450 enzymes such as phenobarbital (cytochrome P450 2A1, P450 2B1) and 3-methylcholanthrene (cytochrome P450 1A1) may be also used.

The livers are removed from the animals after sacrifice at specific time intervals after application of the inducer, minced, homogenized, and centrifuged at 9000 *g* for 15 min. The monooxygenases contained in this S-9 supernatant require NADPH as cofactor, which is normally generated by glucose-6-phosphate dehydrogenase from glucose-6-phosphate by reducing $NADP^+$. Therefore, the S-9-fraction is also supplemented with these two cofactors and with magnesium and potassium salts to yield the final activating system, the S-9 mix. This standard S-9 mix is capable of performing phase-I biotransformation reactions (e.g. oxidations, reductions) but is deficient in most phase-II systems (conjugation reactions). The latter are often involved in detoxification reactions, while phase-I enzymes usually result in toxification of xenobiotics to more electrophilic metabolites. Hence, the S-9-mix may efficiently simulate bioactivation of xenobiotics in the liver, but not detoxification. This may be regarded as an advantage, since it usually increases the sensitivity of the system. On the other hand, the discrepancy between phase-I and phase-II enzymatic activities may be a source of false positive results because the genotoxicity observed in vitro may not reflect the in vivo situation, and detoxification may predominate in the intact animal. The deficiency in conjugation reactions may be partly overcome by adding appropiate cofactors for phase-II conjugation reactions, such asglutathione. The S-9 fraction from other organs may also be prepared and used for the bioactivation of organ specific carcinogens, for example, S-9-fraction of the renal cortex for compounds inducing renal cell tumors. Alternatively, cell-based systems may be used to bioactivate xenobiotics in bacterial mutagenicity assays. Freshly isolated hepatocytes or hepatocytes in primary culture retain the activity of the phase-I and phase-II enzymes of the intact liver and do not require the addition of cofactors for enzyme activity. However, besides greater technical difficulty in obtaining hepatocytes of good quality compared with S-9 fraction, additional problems arise when using intact cells because the electrophiles formed may have a very short half-life and may be trapped and react with macromolecules before diffusing out of the hepatocytes, resulting in false negative results.

An recent elegant approach to overcoming the deficiencies of the bacteria in biotransformation reactions is to clone genes of mammalian biotransformation enzymes into plasmids and to introduce these into tester strains. Although this approach has not been widely used so far, it seems to be a promising method for the future. For example, introducing the gene for *N,O*-acetyl transferase, an enzyme important in the bioactivation of aromatic amines, into *Salmonella typhimurium* TA 98 and TA 100 resulted in an approximately 100-fold in-

crease in the corresponding enzymatic activity in the newly engineered strain. In contrast to the original strains, the *N,O*-acetyl transferase-proficient strains permitted efficient detection of the mutagenic activity of several nitroarenes and aromatic amines. A similar increase in the mutagenicity of bromo- and chloroalkanes was abtained with tester strains expressing a specific human glutathione *S*-transferase isoenzyme [170, 171]. Specific potentials and limitations of these tests follow:

Potentials
- High reliability when testing known carcinogens and noncarcinogens
- Extensive data base available
- High sensitivity
- Information about the mechanism of mutations may be obtained by comparing results in different strains
- Rapid and inexpensive
- May be used as a bioassay to detect mutagenic components in complex mixtures

Limitations
- Some important carcinogens are not active due to specific mechanisms of action (metals, particles, asbestos)
- Data are only qualitative
- Sample must be sterile
- Problems in testing bactericidal chemicals

Experimental Procedure for the Ames Test. Presently three different protocols are widely used for the Ames test: the plate-incorporation assay, the preincubation assay and the fluctuation test.

Plate Incorporation Assay. The basic procedure of the plate incorporation assay is illustrated in Figure 62. Briefly, 2.0 mL aliquots of soft agar overlay medium (Top Agar, 0.6% agar and 0.5% sodium chloride in distilled water) containing a trace of histidine and excess biotin and maintained in the liquid state at 45°C, 100 μL of the tester strain, 20–100 μL of the test compound and, when necessary, 500 mL S9-mix (or another bioactivation system) are added. After mixing, the solution is poured onto dried Vogel–Bonner minimal medium plates. The plates carrying untreated, solvent, and positive (UV light or established mutagen) controls are incubated for 2 d at 37°C. At least ten concentrations of the test compound are usually tested with two plates per concentration.

Preincubation Assay. Some mutagens, particularly those metabolized to short-lived reactive electrophiles, may not be detected in the standard plate incorporation assay due to reaction of the electrophile with constituents of the medium. This type of xenobiotic may be detected more efficiently by using the preincubation assay protocol, in which the bacteria are preincubated with the test compound and S-9 mix in suspension for 30–120 min in the absence of top agar. After the end of this preincubation period, 2.0 mL of soft agar is added to each tube and the reaction mixture is poured into Vogel–Bonner plates, which are incubated as described above.

Fluctuation Test. In the fluctuation test, the number of mutants in a series of small independent replicate cultures is detected. Overnight bacterial cultures are incubated with the test compound and the appropriate metabolizing system (when necessary) in the presence of a trace of histidine. As in the other protocols, this trace of histidine allows a few replication cycles, which are necessary for expression of the mutations following the initial DNA damage. The above mixture is then divided into a large number (usually 50–100) of test tubes or microtiter plates. When the histidine trace is consumed, only revertant cells can grow. Test tubes or wells of the microtiter plates containing revertants become turbid, and the media turn acidic as a result of acid release during growth. This pH drop to 5.2–6.8 can be demonstrated by the color change of a pH indicator such as bromoethyl blue.

Scoring for Colonies in the Plate Incorporation and Preincubation Assay The limited growth of nonrevertant colonies due to the trace of histidine added to enable mutational expression of DNA damage results in a slight background lawn of growth. Therefore, before scoring the plates for revertant colonies, the background lawn should be examined either macroscopically or under the low magnification of a light microscope. At toxic concentrations of the test chemical, the plates appear clear. Also, at toxic concentrations of the test chemical, tiny colonies (minicolonies) may be formed. This happens when, at bactericidal concentrations of the test chemical, very few bacteria survive. The

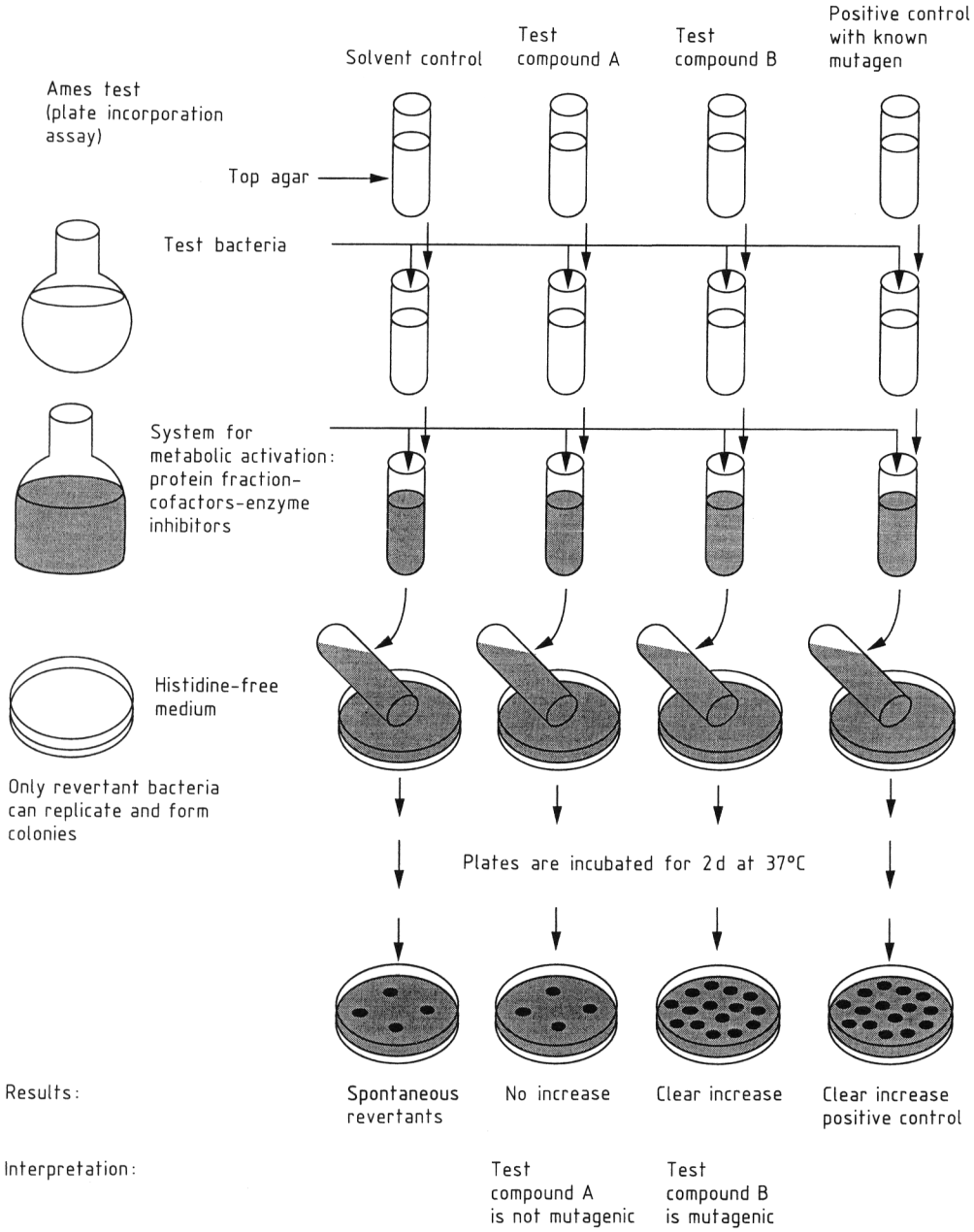


Figure 62. Basic procedure for the Ames test/plate incorporation assay
50 to 200 plates are usually prepared and evaluated per test. At least ten concentrations of the test compound are examined with two plates per concentration level

histidine trace usually added to the system may then be sufficient for formation of these mini-colonies (pseudorevertants). Revertant colonies resulting from gene mutations are clearly larger and can be counted either by hand or with an automatic colony counter.

Evaluation of Results. A typical mutagenic dose–response curve is illustrated in Figure 63. From the linear part of the dose–response curve, the mutagenic potency of the test compound can be calculated and is usually expressed in number of revertants per nanomole of test compound. At

higher, toxic concentrations of the test chemical, the curve may flatten. At concentrations which are very toxic to the bacteria, the number of revertant colonies may start to decline after reaching a maximum. The significance of the results is analyzed with usual statistical methods.

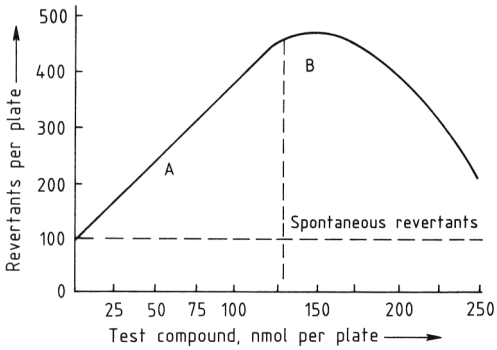


Figure 63. Typical dose – response curve in the Ames test A) At nontoxic mutagen concentrations the number of revertants increases in a dose-dependent manner; B) Higher, toxic concentrations inhibit growth, which is reflected in flattening of the curve followed by decreasing numbers of obtained revertants

Role of the Ames Test in Evaluating Carcinogenic Properties.. The Ames test is a system to detect mutagenicity which has gained great practical importance as a short-term predictive test for potential carcinogenicity of a xenobiotic. Hence, the Ames test may predict the carcinogenic properties of genotoxic carcinogens, but not of nongenotoxic carcinogens. The very high correlation between mutagenicity and carcinogenicity found in previous reports (values of 90–95% were repeatedly estimated) are certainly not correct. One reason for this overestimation may be that in previous decades mainly structurally alerting chemicals (xenobiotics carrying functional groups present in other carcinogens and known or suspected to be metabolized to electrophiles) were tested for long-term rodent carcinogenicity, and most of these structurally alerting carcinogens are mutagenic. In contrast, more recently, more important environmental pollutants and occupational hazardous chemicals have increasingly been tested for carcinogenicity, independent on their chemical structure. As a result, an increasing number of carcinogenic compounds that do not react directly with DNA to cause mutations have been found. These xenobiotics are thought to act via additional and (unfortunately) only partly defined so,called epigenetic mechanisms (i.e., not involving genetic changes). Nongenotoxic carcinogens cannot be detected in the Ames test. In contrast to the genotoxic carcinogens, which are usually active in two species and/or at multiple sites, nongenotoxic carcinogens are often active in one species and at a single site, for example, branched chain hydrocarbons in the male rat kidney or peroxisome proliferators in the mouse liver. This is confirmed by comparing the carcinogenicity results with the bacterial mutagenicity. While more than 70% of the two-species/multiple-site carcinogens are positive in the Ames test, only 40% of the one-species/single-site carcinogens exert bacterial mutagenicity. Therefore, a reasonable evaluation of the results obtained in bacterial mutagenicity systems can only be conducted by taking into consideration additional information on the chemistry, toxicokinetics, biotransformation, and biological effects of the test compounds.

Presently, the Ames test is an important part of a screening battery for possible carcinogenic properties in the course of the development of new pharmaceutical drugs and chemicals. Depending upon the intended use of the chemical, the development may be interrupted if a compound is clearly positive in the Ames test and in an additional short-term test, often the in vivo micronucleus test (see Section 4.9.5.3). With this “decision-point approach”, the number of unnecessary life-long bioassays in rodents is significantly reduced and large amounts of money and time may be saved.

4.9.1.2. Mutagenicity Tests in *Escherichia coli*

The *E. coli* tester strain WP2 and additional tester strains subsequently developed originate from the wild-type *E. coli* B strain; all tester strains are tryptophan(trp)-auxotrophic. For example, the *E. coli* WP2 trp E group has a terminating mutation at an AT base pair of the trp E gene which codes for anthranilate synthetase, an enzyme of the tryptophan biosynthetic pathway. Hence, the *E. coli* mutagenicity assays, like the Ames test, detect reversion of the deficiency to

synthetize tryptophan. The *E. coli* tester strains have umuDC^{+} genes coding for the error-prone repair system, and their membrane structure permit the passage of many large molecules. The experimental procedures and the inherent advantages and limitations are practically identical to those of the *Salmonella typhimurium* test. However, the *E. coli* test systems are less well validated than the Ames test, and this renders the selection of appropiate strains for the investigation of the mutagenicity of a specific xenobiotic and the interpretation of the obtained results more difficult.

4.9.1.3. Fungal Mutagenicity Tests

The most widely used species is *Saccharomyces cerevisiae*; the test detects reversion of the isoleucine auxotrophy induced by exposure to mutagens, in analogy to the Ames test.

4.9.2. Eukaryotic Tests for Mutagenicity

Although prokaryotic systems are fast, inexpensive and versatile, there is a requirement for mammalian test systems due to the important differences between prokaryotic and eukaryotic organisms in structure and function of the genome and in biotransformation and transport of xenobiotics. The eukaryotic systems for detecting mutations include tests in the fruit fly *Drosophila melanogaster*, in many different types of cultured mammalian cells, and in the intact animal in vivo.

4.9.2.1. Mutation Tests in *Drosophila melanogaster*

The fruit fly *Drosophila melanogaster* has a short generation time of 10 d and a similar cellular and chromosomal structure and function to mammalian cells. In addition, the genome of the fly is well characterized, and *Drosophila* species can perform many of the phase-I and phase-II reactions occuring in mammals. The *Drosophila* sex-linked recessive lethal test was a popular screening system in the 1970s and was used to screen many hundreds of compounds. However, since then the popularity of the test has waned drastically because of its poor performance in international collaborative trials that investigated the utility of mutagenicity assays as predictive tools. Another *Drosophila* test, devised in the 1980s, the somatic mutation and recombination test (SMART) seems promising but has not been sufficiently validated. Since both tests are not widely used for screening, the experimental procedures are not be described here (the interested reader may find them in reference [156]). Some potentials and limitations of the mutation tests in *Drosophila melanogaster* follow:

Potentials
- Large number of test organisms can be raised
- Metabolic activation endogenous
- Genotoxic effects evaluated in germ cells
- Large data base on mutagens

Limitations
- Some known carcinogens such as polycyclic hydrocarbons respond poorly
- Problems with toxic compounds (insecticides)

4.9.2.2. In Vitro Mutagenicity Tests in Mammalian Cells

Many short-time mutagenicity tests in mammalian cell lines have been developed. These systems have the advantage of determining genotoxic effects in relevant cell types and may permit the study of mechanisms of carcinogenicity. The potentials and limitations of these in vitro tests follow.

Potentials
- Full range of mutational responses may be observed
- Low costs when compared to in vivo assays
- Respond to particulates and other types of compound not detected in the Ames test
- More relevant end point for mammals

Limitations
- Most cell lines used are transformed cells and do not represent "normal" cells
- Only specific loci from the entire genome are monitored
- Most cell lines used are deficient in biotransformation enzymes

In contrast to popular bacterial assays that detect reversion mutations, the commonly used

mammalian mutagenicity assays are based on the detection of forward mutations. A large number of cells is treated with the test compound. After a certain period of time, the cells are exposed to a toxic agent that is lethal to all cells not carrying mutations (i.e., only mutated cells can survive). Cultured mammalian cells are normally diploid and have two copies of each gene. Hence, recessive mutations may be missed if a normal copy is present on the homologous chromosome because the probability that a mutagenic compound alters genes is very low. Therefore, mutations are assessed in genes on the X chromosome in male cells, where only one copy of the gene is present. Alternatively, heterozygous cells are used, in which one copy of the gene is already inactivated.

Mammalian cells have genes that allow the cell to salvage nucleotides from the surrounding medium. These genes, although not essential for cell survival, save cellular energy, since the cell need not synthesize these molecules from simple precursors by energy-consuming pathways. If the medium is supplied with altered nucleotides, the normal (not mutated) cell will incorporate them into the DNA, and this will result in cell death. However, if this salvage property is lost due to mutation, the mutated cells are not able to incorporate the toxic nucleotides from the surrounding medium and will survive and form colonies which can be detected as a parameter of genotoxicity. Although a gene may be inactivated by a mutation, the mRNA and the corresponding enzyme produced prior to the mutational event may be present for some time after exposure to the mutagenic test compound. Therefore, the cells have to be left for some time before challenging with the toxic nucleotide, this period is called expression time.

Basically, two genes of the salvage pathway are utilized in mutagenicity assays: the hypoxanthine–guanine phosphoribosyltransferase (HGPRT) gene and the thymidine kinase (TK) gene. The HGPRT gene is located on the X chromosome in humans and in the Chinese hamster, from which many useful cell lines have been developed. The genetic changes detected in the HGPRT gene as the target site are mainly point mutations because chromosomal deletions usually extend to flanking regions of the target gene, which may contain essential genes. Since only one copy of these essential genes exists in male cells, this results in cell death so that the cells in which the HGPRT gene is inactivated by deletion are usually lost for the test. This is not the case with the TK gene, because it is autosomal and heterozygous cells (TK^+/TK^-) are used in the assay. Here, both point mutations and changes at the chromosomal level can be detected. The HGPRT gene is the target gene in the Chinese hamster CHO and V 79 cell lines, while mouse lymphoma L 5178 Y $TK^+/^-$ cells detect mutations at the TK locus. The mechanistic background of these tests is shown in Figure 64.

The use of Chinese hamster cell lines for mutagenicity screening is limited due to low sensitivity. Chinese hamster cell lines grow on monolayers and, owing to metabolic cooperation, only a relatively small cell population can be used. In contrast, L5178Y $TK^-/^-$ cells grow in suspension, and the system is not impaired by the problems of metabolic cooperation, because intercellular bridges do not occur. As described above, a wide variety of genetic events including gene mutations, recombinations and mitotic nondisjunction can result in the formation of the $TK^+/^-$ genotype from the heterozygote $TK^+/^-$. Two protocols have been devised for mutation assays with mouse lymphoma L5178Y cells:the plate test in soft agar and the fluctuation test in suspension. For dose-finding, a preliminary cytotoxicity assay is usually conducted when testing new compounds. In this assay, the cloning efficiency (for the plate test) or the relative suspension growth (for the fluctuation test) is determined. The highest test concentration for the mutation assay is usually the concentration that reduces cloning efficiency or suspension growth to approximately 10–20% of the control values. In addition, a moderate concentration causing survival reduction to 20–70% and a low concentration ($> 70\%$ survival) are also used. After treatment with the test compound (3–7 h; in the presence or absence of an exogenous metabolic system, e.g., S-9 mix) the cells are incubated for 2 d for mutation expression before exposure to trifluorothymidine for 10–12 d. In the presence of trifluorothymidine, only mutant cells can survive. Thus, the relative growth of the cells at the end of the experiment can be used as a parameter for mutagenicity.

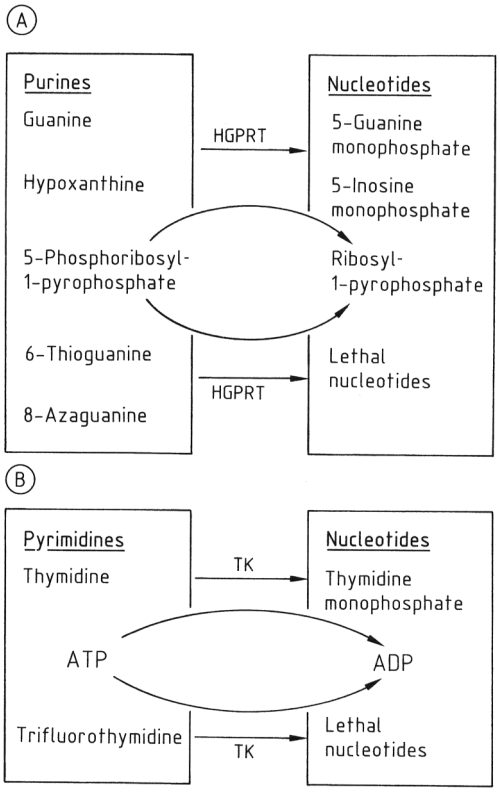


Figure 64. Assessment of mutagenicity in mammalian cells
A) The V 79/HGPRT (hypoxanthine – guanine phosphoribosyltransferase) assay; B) The L 5178 Y TK^{+}/$^{-}$ (thymidine kinase) mouse lymphoma assay
Mutant cells do not form the lethal nucleotides and survive in the presence of 6-thioguanine or trifluorothymidine.

4.9.3. *In Vivo* Mammalian Mutation Tests

In contrast to the well-established and widely used micronucleus tests in rodents (see Section 4.9.5.3), the existing in vivo tests to detect gene mutations are not used for routine genetic toxicology testing; therefore, only brief descriptions are given here (for exact experimental procedure, see [156]).

4.9.3.1. Mouse Somatic Spot Test

The mouse somatic spot test detects the inactivation of genes at a set of heterozygous loci controlling hair pigmentation. Inactivation can be induced by a wide spectrum of genetic alterations ranging from gene mutations to major chromosomal changes.

4.9.3.2. Mouse Specific Locus Test

The mouse specific locus test consists of treating parental mice homozygote for a marker gene controlling coat pigmentation, intensity, and pattern or size of the external ear. Treated mice are mated with a tester stock that is homozygous recessive at the marker loci. The resulting F_1 generation is normally heterozygous at the marker genes and therefore expresses the wild-type phenotype. In the case of mutations at any of these genes, the F_1 offspring express the recessive phenotype. The mouse specific locus test has a comparatively low sensitivity, thus requiring that many thousands of newborns be scored. However, it remains an important method for investigating heritable mutations.

4.9.3.3. Dominant Lethal Test

The dominant lethal test assesses embryonic death resulting from genetic changes in parental germ cells. The procedure is based on observing the viability of uterine implantations. Implantations that die at an early stage form a deciduoma or mole. The genetic event resulting in early death is predominantly chromosomal damage in the parental germ cells resulting in a dominant lethal mutation. Although the assay was popular in the 1960s and 1970s as a screen for germ cell damage, it is not widely used at present because it is considered relatively insensitive.

4.9.4. Test Systems Providing Indirect Evidence for DNA Damage

4.9.4.1. Unscheduled DNA Synthesis (UDS) Assays

Two major DNA repair mechanisms have been identified in mammalian cells. The first mechanism involves direct reversal of DNA damage, such as cleavage of pyrimidine dimers that have been induced by UV light or removal of potentially mutagenic methyl groups from the O^6 position of guanine by O^6-methylguanine-DNA methyltransferases. The second mechanism recognizes damaged DNA bases, removes them after nicking the DNA backbone, fills the gap formed with the correct bases using the opposite strand as a template, and finally seals the second strand break.

The most widely used method to study DNA repair is the "unscheduled" DNA synthesis assay. The principle of the UDS assay consists in detection of the incorporation of radioactive DNA bases or chemical analogues of nucleotides such as bromodeoxyuridine into DNA in the course of excision repair after damaged bases have been removed. This unscheduled DNA synthesis induced by DNA damage must be distinguished from the semiconservative DNA synthesis during DNA replication. DNA replication can occur in the same cells that undergo repair or, alternatively, in other cells of the test population and usually contributes to an incorporation of radiolabeled base or nucleotide analogue in concentrations several orders of magnitude higher than repair. Three approaches are currently available to overcome this problem and to detect selectively DNA repair-dependent incorporation.

1) Use of quiescent cell populations, that do not replicate
2) Suppression of DNA replication by incubation in serum-reduced and arginine-depleted medium and pretreatment with the ribonucleotide reductase inhibitor hydroxyurea
3) Evaluation of radioactivity by autoradiography of single cells, which allows the distinction between heavily labeled replicating cells and lightly labeled DNA-repairing cells

The UDS assay can be conducted in vitro and in vivo. The following description briefly outlines the in vitro assay in cultured mammalian cells. After inhibition of DNA replication by serum reduction, arginine depletion, and treatment with hydroxyurea (if replicating cell lines are used), the cells are exposed to the test compound in presence of radiolabeled thymidine. Among the various cell types used, primary cultures of rat hepatocytes are often preferred to permanent cell lines, because their biotransformation capabilities are closer to those of liver cells in vivo. In addition, primary cultures of hepatocytes do not divide, so that no suppression of cell proliferation is necessary.

Radioactivity incorporation can be measured by scintillation counting of the isolated DNA or by autoradiography of whole cells. For determination of UDS in vivo, the animals are treated with the test compound and radiolabeled thymidine (most often applied by implanted minipumps). Radioactivity in DNA is determined after isolation of the DNA. Alternatively, a combined in vivo/in vitro approach may be used. In this case, in vivo treatment of the animals is followed by isolation of cells from the appropiate organs and labeling of the cells with the radioactive nucleotide in vitro. Assessment of radioactivity incorporation after in vivo treatment is usually conducted by autoradiography.

Several factors influence the interpretation of UDS data. Detection of excision repair demonstrates that the test compound has caused repairable damage to DNA, but does not answer the question whether this has any significance for the mutagenic potential of the chemical. This is very important, because measurements of UDS are averages over all cells of the study population, whereas mutation is a rare event in an individual cell. In addition, extranuclear events such as mitochondrial toxicity resulting in oxidative stress and increased production of reactive oxygen species or increased calcium concentrations in the nucleus and activation of endonucleases may also influence indirect DNA damage and induction of UDS.

4.9.4.2. Sister-Chromatid Exchange Test

Sister- chromatid exchanges are reciprocal exchanges between sister chromatids thought to occur at homologous loci. Although detection of sister-chromatid exchanges can also be conducted after in vivo treatment, the test is widely applied in established cell lines in vitro (Chinese hamster cell lines, human fibroblast cell lines) and in freshly isolated human lymphocytes. Any cell type that is replicating or can be stimulated to divide can be used. The test is carried out essentially according to the following basic procedure. Cells in exponential growth are usually exposed simultaneously to 5-bromo-2′-deoxyuridine and the test compound for a period equivalent to approximately two cell cycles (incubation time depends on the cell type used). In the final 1–2 h of incubation, a spindle inhibitor (colchicine or demecolcine) is added to arrest the cells in metaphase. For detection of sister-chromatid exchanges, the two chromatids are differentially stained with a fluorescent dye such as Hoechst 33258 plus Giemsa. With this method, sister-chromatid exchanges appear as color change on one chromatid (Figure 65).

The sister-chromatid exchange test is one of the quickset, easiest, and most sensitive methods for detecting genetic damage. A large number of compounds have been evaluated with this method, which is usually part of the test battery for genotoxicity screening.

The problem is that the precise genetic event detected by this test is basically not known. sister-chromatid exchanges are obviously not related to chromosomal aberrations since they often fail to detect potent clastogens. They relate more to gene mutations, although there are examples of compounds that clearly induce sister-chromatid exchanges in the absence of mutations as well as the converse. Despite the uncertainty resulting from the unknown mechanism, the sister-chromatid exchange test is very useful because it detects important groups of potentially carcinogenic compounds such as alkylating agents and nucleotide analogues, as well as compounds inducing DNA single-strand breaks or acting through DNA binding.

4.9.5. Tests for Chromosome Aberrations (Cytogenetic Assays)

4.9.5.1. Cytogenetic Damage and its Consequences

In addition to gene mutations, most tumor cells investigated thus far exhibit structural and/or numerical chromosomal aberrations. Chromosomal aberrations such as deletions and translocations may result in activation of proto-oncogenes or inactivation of tumor-suppressor genes. In certain cancers of the lymphatic system (human Burkitt's lymphoma and mouse plasmocytoma), the normal proto-oncogene *c-myc*, which is involved in the regulation of cell proliferation, is translocated to the immunoglobulin locus. Immunoglobulin genes show very high transcription rates during development of B lymphocytes. The translocated *c-myc* is subjected to the same control mechanisms, resulting in inappropriately elevated expression of the gene and increased cell proliferation. In addition to these quantitative changes, chromosome aberrations may result in alterations of structure and function of cellular proteins. In many cases of human chronic myeloid leukemia, a piece of chromosome 9 carrying the proto-oncogene *c-abl* undergoes reciprocal translocation with a piece of chromosome 22. This results in joining of the proto-oncogene *c-abl* with a gene on chromosome 22 and production of a fusion protein encoded by both DNA sequences.

During cell division, chromosome segregation depends upon the functional integrity of the proteins of the spindle apparatus, that correctly divides the replicated genome into the two new nuclei. Impairment of this process, i.e. by chemical damage to the spindle apparatus, may result in nondisjunction, which means that both

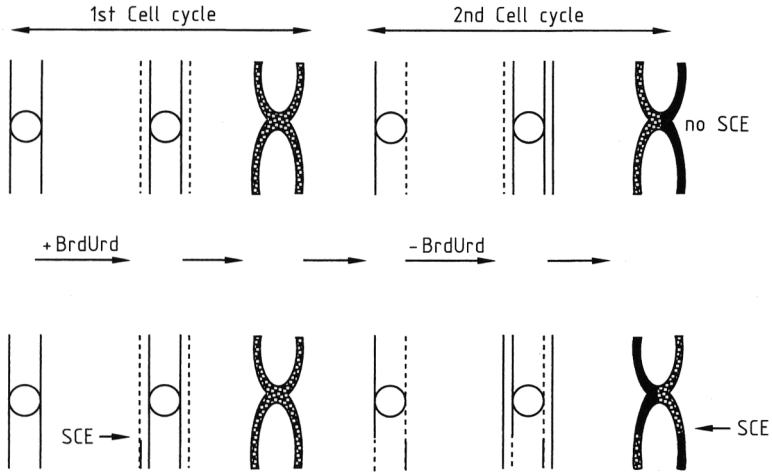


Figure 65. Sister-chromatid exchange (SCE)
The exchange is visualized by treating cells with 5-bromo-2′-deoxyuridine (BrdUrd) during two DNA replication cycles. The distribution of BrdUrd between chromatids is then determined by staining with a fluorescent dye and UV irradation.

copies of a particular chromosome move into one daughter cell while the other receives none. Approximately 10% of the tumor types investigated so far are monosomic or trisomic for a specific chromosome. Aneuploidy is also an important cause of severe birth defects. Trisomy or monosomy of large chromosomes usually leads to early death of the embryo; trisomy of smaller chromosomes may allow the embryo to survive, but the newborn shows severe anatomical abnormalities and major physiological impairment. The most common syndrome of this group is Down's syndrome, which results from trisomy of chromosome 21.

Hence, structural and numerical chromosome aberrations may have detrimental effects on the health of the affected persons, and the following sections describe assays to detect chromosome aberrations in vitro and in vivo.

4.9.5.2. In Vitro Cytogenetic Assays

In the in vitro cytogenetic assays, proliferating cultured cells are treated with the test compound, and chromosomal aberrations are investigated after one or more cell cycles. Although cytogenetic assays can, in principle, be carried out in any cell type, Chinese hamster cell lines and human peripheral blood lymphocytes from healthy donors are generally used. In contrast to the spontaneously dividing Chinese hamster cell lines, peripheral blood lymphocyte cultures must be stimulated to divide by a mitogenic agent, such as phytohemagglutinin. Treatment is performed about 44 h after phytohemagglutinin stimulation, when the cells are proliferating. At least three doses of the test compound should be investigated, with the highest being in the low cytotoxic range. The usual recommendation is to treat the cells for ca. 1.5 normal cycles times, which is 15 h for Chinese hamster cells and 12–14 h for human peripheral lymphocytes. Many authors recommend longer treatment periods because some chemicals induce a mitotic delay at clastogenic doses (i.e., they increase the duration of the cell cycle). At the end of treatment, cells are induced to accumulate in the metaphase stage of mitosis with a spindle inhibitor such as colchicine or demecolcine, and at least 200 cells per treatment group are scored under the microscope for chromosomal aberrations. Chromosome breaks, fragments, and exchanges are considered specific structural aberrations, whereas gaps are usually excluded from the quantitation because the mechanism underlying their formation is not understood. Chromosome pulverization (complete fragmentation in small pieces) may also be regarded not as a parameter of clastogenicity, but rather as an indicator of severe cytotoxicity and cell death induced by the test chemical. Chemicals that are clastogenic in vitro at noncytotoxic concentrations are likely to be clastogenic in vivo. When bias resulting from lack of or insufficient biotransformation in the in vitro situation can be excluded, negative results in the in vitro assay provide a strong indication for absence of in vivo clastogenesis.

4.9.5.3. *In Vivo* Cytogenetic Assays

Induction of chromosome aberrations can be detected in intact animals either by examination of metaphases as described above for cultured cells or by the formation of micronuclei. Rats and Chinese hamsters are usually employed in metaphase analysis, while mice are commonly preferred in the widely used micronucleus test in bone marrow cells.

Rodent Micronucleus Test. The rodent micronucleus assay is an important part of the test battery for genotoxicity and is usually included in the toxicity evaluation of both pharmaceuticals and chemicals. The assessment of micronuclei is commonly conducted in polychromatic erythrocytes of bone marrow after treatment of young mice for six to eight weeks. Induction of numerical chromosome aberrations or chromosome breaks in the immature erythroblast of the bone marrow results in the formation of a micronucleus after cell division because the chromosome fragments are not participating in the chromosome segregation accomplished by the spindle apparatus. In the course of the physiological maturation of the red blood cells, the main nucleus is extruded from the erythroblast, while the micronucleus remains in the cell and can be detected in the young polychromatic erythrocyte after staining (Figure 66). The lack of nucleus in these bone marrow cells facilitates scoring for micronuclei under the microscope;

however, peripheral blood cells and liver cells have also been used.

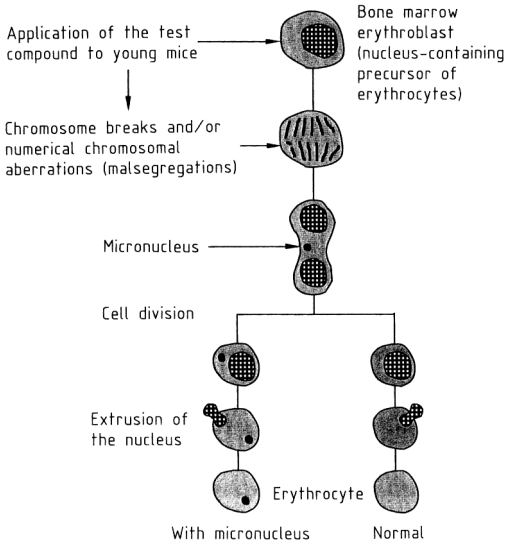


Figure 66. Mechanism of micronucleus formation in bone marrow erythroblasts

Usual treatment protocols include application of a single dose of the test compound and harvesting of bone marrow cells after 24, 48, and 72 h; or, alternatively, multiple applications (two or three doses 24 h apart) followed by a single harvest another 24 h after the last dose. The bone marrow is aspirated with a needle from the proximal end of the femur shafts and flushed with serum to obtain a homogeneous cell suspension, and after staining, the number of micronucleated immature polychromatic erythrocytes (not the number of micronuclei) is scored under a microscope. The immature erythrocytes stain bluish and can be distinguished clearly from the orange-red mature erythrocytes. Only tests with positive and negative control values within the historical control value of the laboratory are considered adequate. The minimum number of cells scored depends on the spontaneous frequency of micronuclei; 2000 cells should be scored if the control incidence is $< 0.2\%$ [172]. The ratio of polychromatic immature erythrocytes : monochromatic mature erythrocytes indicates the integrity of proliferation and differentiation processes in the bone marrow and is used as a measure of general toxicity. If the test compound has been administered at the maximum tolerated dose (as determined in the subacute toxicity test) and no increase in micronucleated erythrocytes can be detected, it is classified as negative for clastogenic activity in vivo. Clear positive results in the in vivo assay indicate clastogenic potential also in humans.

Rodent Bone Marrow Metaphase Analysis. Metaphase analysis is usually carried out in rats at 6, 24, and 48 h after administration of a single dose or alternatively at 6 and 24 h after administration of multiple doses at 2 to 4 h prior to sampling. The animals are treated with spindle inhibitor such as colchicine and demecolcine to induce accumulation of cells in the metaphase. Metaphase analysis is much more time- consuming than scoring for micronuclei; therefore, only 50 cells per animal (500 per experiment with five male and five female animals) are usually analyzed [172]. In contrast to the rodent micronucleus test, the bone marrow metaphase analysis is not automatically included in the general screening scheme for genotoxicity; it is performed only when specific questions arise or for research purposes.

4.9.6. Malignant Transformation of Mammalian Cells in Culture

The term transformation in the present context means that a cultured cell line has taken on one or more morphological and/or malignant changes to give tumor cells. Among the various changes observed by exposure of cell lines to carcinogenic compounds, the following are two clearly established parameters indicating morphological transformation:

1) Inducation of anchorage independence (i.e., cells become capable of forming colonies in soft agar)
2) Loss of contact inhibition resulting in a tendency to grow in a piled-up criss-crossed pattern and to form foci

The term malignant transformation indicates that a cell line is capable of producing an invasive tumor in a suitable host. Hence, not every morphologically transformed cell line has necessarily undergone malignant transformation. On the other hand, human cancer cells are by definition malignantly transformed because they may metastasize under appropriate conditions.

The vast majority of cell transformation studies have been carried out with Syrian hamster cells [i.e., Syrian hamster embryo (SHE) cells and baby hamster kidney (BHK) cells] and with human fibroblasts.

SHE cells, isolated from 13-day-old embryos, can be induced to form piled-up criss-crossed colonies (foci) by treatment with carcinogens. Cells derived from these foci can produce tumors in athymic mice after extended subculturing (35–70 population doublings). Hence, the induction of malignantly transformed tumorigenic cells is a laborious, time- consuming procedure with several inherent problems (e.g., contamination of the plates in the course of the multiple passaging steps over several months). Therefore, the tendency developed to establish morphological transformation that can be induced in a comparatively short time as a short-term test for carcinogens. However, anchorage-independent cells showing criss- crossed growth pattern are usually not carcinogenic in animals. In addition, interlaboratory comparative elaboration studies indicated that scoring may be arduous and subjective, and in many cases the lack of dose-response and difficulties in obtaining consistently reproducible results in repeated assays may occur, even in the same laboratory [173].

Malignant transformation of cultured cells is currently an important tool to investigate the molecular mechanisms, i.e. mutations in oncogenes and tumor suppressor genes likely involved in the carcinogenic process. However, the use of cell transformation assays for screening purposes and for human risk assessment is very limited.

Two in vitro tests are required to provide a base level of information on the mutagenic potential of a chemical. These are a gene mutation test in bacteria and an in vitro mammalian cell test capable of detecting chromosome aberrations. For chemicals with significant toxicity to bacteria, an *in vitro* mammalian cell gene mutation test can be used as an alternative first test.

There are various options for selection of further test procedures in mammalian cells. An in vitro chromosome aberration test, i.e., a cytogenetic assay for structural chromosome aberrations using metaphase analysis will povide information on potential aneugenicity by recording the incidence of hyperdiploidy, polyploidy, and/or modification of mitotic index (e.g., mitotic arrest). A mouse lymphoma assay (L5178Y cells, TK locus) may detect gene mutations and structural chromosome aberrations but is not sufficiently sensitive for the detection of aneugens. These systems may be combined with an in vitro micronucleus tests, which is capable of detecting structural chromosome aberrations as well as aneuploidy.

4.9.7. In Vivo Carcinogenicity Studies of Limited Duration

In vivo tests of limited duration provide evidence for carcinogenicity in the whole animal in a short period (i.e., 52 weeks or less) compared to the lifelong bioassay. The experimental procedure usually involves administration of several doses of a known initiating carcinogen for a specific target organ to investigate promoting effects of the subsequently administered test compound. Alternatively, a known potent promoter for a specific tumor can be used to facilitate detection of initiating properties of the test compound. Also, many limited bioassays currently established or being developed aim to predict carcinogenicity by detecting cell lesions that consistently precede the appearance of the relevant tumor (i.e., induction of altered foci in the liver). In vivo carcinogenicity studies of limited duration are not automatically included in the usual test battery for carcinogenicity and are not accepted by regulatory authorities as a replacement for the conventional lifelong carcinogenicity study. The decision to carry out limited bioassays is made individually according to the available and required information on the test compound. Therefore, the present chapter includes only a brief description of the limited bioassays currently available.

4.9.7.1. Induction of Altered Foci in the Rodent Liver

In the course of rat liver carcinogenesis, cell foci exhibiting specific biochemical alterations precede the formation of liver tumors. These foci have, for example, abnormal concentrations of γ-glutamyl transpeptidase, glucose-6 phosphatase, adenosine triphosphatase, and the pla-

cental form of glutathione *S*-transferase and, unlike normal liver parenchyma, do not accumulate iron following iron loading. These parameters allow reliable and objective histochemical identification of preneoplastic foci as soon as three weeks after the initiation of treatment with known carcinogens. The yield is usually highest by the 12th to 16th week of exposure; therefore, the recommended approach is exposure for 12 weeks to the test compound with subcutaneous injection of iron during last two weeks to produce the iron load [174].

4.9.7.2. Induction of Lung Tumors in Specific Sensitive Strains of Mice

Certain strains of mice (e.g., the A/Heston) exhibit a high spontaneous incidence of lung tumors and are extremely sensitive to pulmonary carcinogens such as polycyclic aromatic hydrocarbons, certain nitrosamines, aflatoxin B_1 ethyl carbamate, hydrazines, and certain alkylating agents. The sensitivity of the test is highest 30 to 35 weeks after exposure initiation. Extension of treatment for a longer period is not recommended, because after 35 weeks the spontaneous lung tumor incidence increases rapidly in control animals as well, resulting in decreased sensitivity of the test.

4.9.7.3. Induction of Skin Tumors in Specific Sensitive Strains of Mice

The carcinogenic activities of several chemicals and crude mixtures can be readily revealed by their continuous application to the skin of a highly sensitive mouse strain, the Sencar mouse. Tars from coal, petroleum, or tobacco, as well as the pure polycyclic aromatic hydrocarbons and their congeners contained in such mixtures, give clearly positive results in this limited bioassay; in contrast, some arylamines and other established carcinogens do not elicit a positive response in this mouse skin test. These discrepancies are due mainly to differences in toxification or detoxification reactions between the systemic and the local applications. The mouse skin contains specific cytochrome P450 enzymes and peroxidases that can bioactivate polycyclic aromatic hydrocarbons, hence the differences in response. Therefore, this test cannot be considered a reliable predictor of the potential carcinogenicity of a xenobiotic or of human risk.

4.9.8. Methods to Assess Primary DNA Damage

4.9.8.1. Alkaline Elution Techniques

Many biochemical and analytical procedures are available to detect and quantitate the damage to DNA after contact with xenobiotics. Some of these procedures are very sensitive and thus permit detection and quantitation of DNA damage or DNA modifications after application of relevant doses of carcinogens to animals and after occupational or environmental exposure of humans.

The alkaline DNA filter elution was developed based on the observation that the rate at which large DNA single-strands pass through a membrane filter under alkaline denaturing conditions depends on the length of the strands. A broad spectrum of DNA damage types – both direct and indirect effects caused by effects of the toxicant on cellular function – can be detected with this techniques. The indirect effects include DNA single-strand breaks, DNA–protein crosslinks, and interstrand crosslinks (DNA–DNA).

The basic procedure (Fig. 67) operates essentially as follows: After treatment of cells in vitro or isolation of cells from an organ or a tissue of an animal treated in vivo, cell samples are placed onto membrane filters and lysed with a detergent- containing solution. This lysis solution is allowed to flow through the filter, thus removing most of the cellular protein and RNA, the intact DNA of cellular chromatin being retained on the filter. An elution solvent with a pH generally >12.0 is then pumped slowly through the filter to disrupt the hydrogen bonds between DNA strands. Treatment-induced DNA single-strand breaks produce DNA fragments with reduced molecular masses, thus increasing the rate at which DNA passes through the filters, whereas DNA–DNA or DNA–protein crosslinks decrease the rate compared to untreated controls. The amount of DNA eluted can be quantified either by using radiolabeled cell populations (by pretreating the cells with [^{3}H]thymidine) or by

fluorogenic DNA-reactive compounds. The major limitations of the test are essentially the same as described in Section 4.9.4. DNA strand breaks do not necessarily indicate mutagenicity or interaction of a xenobiotic with DNA; they may also result from extranuclear damage such as increased production of reactive oxygen species due to impairment of mitochondrial functions. Hence, although a vast amount of data exists on the ability of mutagens and carcinogens to induce single-strand breaks, the test has only limited value as a screening method for mutagenicity.

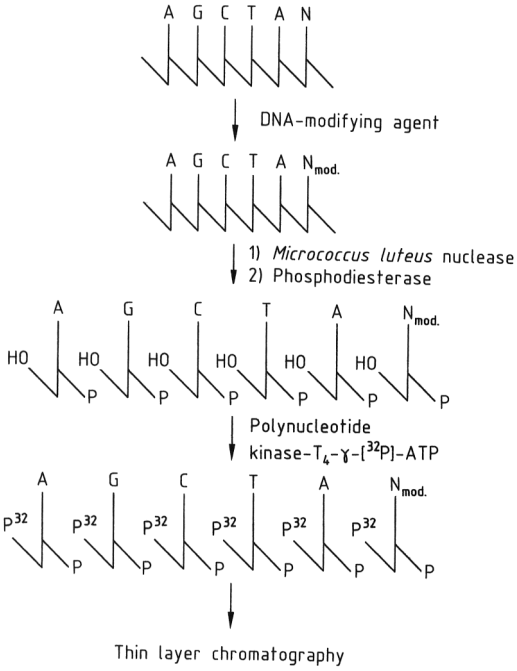


Figure 67. The ^{32}P-postlabeling procedure for detection of DNA damage ($N_{mod.}$ = modified deoxynucleotide)

4.9.8.2. Methods to Detect and Quantify DNA Modifications

The increased sensitivity of analytical methods has resulted in the development of several sensitive and selective methods to detect and quantify modifications of DNA bases induced by xenobiotics. These methods often rely on sophisticated chromatographic techniques to separate unchanged DNA constituents from modified DNA bases.

High-performance liquid chromatography (HPLC) with fluorescence detection may be used to detect and quantify DNA adducts formed from highly fluorescent xenobiotics, such as aflatoxin$_{B1}$ and polycyclic aromatic hydrocarbons. In addition, some DNA adducts of xenobiotics are highly fluorescent, such as alkylations of the N^7 atom of guanosine [175] and cyclic derivatives formed from vinyl chloride [176]. HPLC with electrochemical detection may detect oxidative DNA modifications, such as 8-hydroxydeoxyguanosine with high sensitivity and has been used to study DNA damage by cellular aging [177].

Gas chromatography/mass spectrometry (GC-MS) techniques are widely used in the detection and quantitation of DNA modifications. After derivatization to form volatile derivatives, DNA adducts formed by methylating and ethylating agents can be detected by GC-MS with selected-ion monitoring [178]. Due to the low detection limit (attomole range), GC-MS coupled with chemical ionization and negative-ion detection (after electrophore labeling with. e.g. pentafluorobenzyl bromide) has been applied in DNA adduct monitoring [179, 180]. However, the procedures are very time consuming and prone to artefact formation. At present, due to much simpler sample workup and high sensitivity LC-MS/MS methods promise to be useful for detecting DNA modifications.

A widely used method to detect DNA adducts of bulky organic substituents, which can not be transformed to volatile derivatives, is the ^{32}P-postlabeling technique [181]. The method can be widely used because it does not require the use of radiolabeled xenobiotic and sophisticated and expensive mass spectrometry. Due to the availability of ^{32}P-labeled adenosine triphosphate with high specific activity and the possibility of concentrating DNA adducts, this procedure may detect adduct frequences as low as one adduct in 10^{10} nucleotides. The method involves isolation of DNA from an animal or cells treated with the xenobiotic, enzymatic hydrolysis of the DNA to the 3′-nucleoside monophosphates and enzymatic phosphorylation to the 3′,5′-nucleoside diphosphates with ^{32}P-labeled adenosine triphosphate as phosphate donor. The obtained mixture of ^{32}P-labeled nucleosides and modified nucleosides is then separated by multidimensional TLC. Modified nucleosides are de-

tected by placing the TLC plates on radiation-sensitive film; quantification can be performed by liquid scintillation spectrometry after cutting the adduct spots from TLC plates. Formation of artefacts and the poor resolution of the employed TLC method are problematic.

Several procedures to detect DNA adducts based on immunological methods have also been developed. These procedures have the advantage of being rapid and simple, but they are usually selective only for a specific type of adduct and require a time-consuming procedure to generate the antibody. In addition, sensitivity is often insufficient, quantification of adducts is complicated, and cross-reactivity generates artefacts.

Modern LC-MS/MS methods have sufficiently low detection limits to detect DNA modifications in a concentration range relevant for animal toxicity studies and do not require time-consuming sample preparation. Therefore, these methods have major potential for widespread use in the detection and quantitation of DNA modifications.

4.9.9. Interpretation of Results Obtained in Short-Term Tests

Although no single rule applies in every case and each test compound should be evaluated individually, some general recommendations may be useful for a reasonable interpretation of results from short-term tests. Positive results in one in vitro test for mutagenicity (i.e., the Ames test) and one in vivo test (i.e., the rodent bone marrow micronucleus assay) indicate potential carcinogenicity. The test compound is highly suspect for potential carcinogenicity if clear-cut, dose-dependent evidence for genotoxicity in more than one in vivo and one in vitro test has been obtained, especially if the compound or its metabolites are structurally alerting for DNA reactivity.

This is supported by a recent evaluation on the activity of known human carcinogens in the *Salmonella* mutagenicity test and the rodent bone marrow micronucleus test [182]. As shown in Table 34, most of the human carcinogens identified so far are positive in these popular tests. Apart from hormones, no nongenotoxic organic chemical has been shown to cause cancer in humans thus far. In contrast, since the late 1980s an increasing number of organic chemicals has emerged that are clearly carcinogenic in rodents but are not mutagenic in *Salmonella* or clastogenic in rodent bone marrow. In addition, most of these nongenotoxic rodent carcinogens (and their metabolites) do not exhibit structural alerts for DNA reactivity. The reason for the differences between the epidemiological data in humans and the chronic rodent bioassays are not

Table 34. Mutagenicity in Salmonella typhimurium and clastogenicity in rodent bone marrow of known human carcinogens [191]

Human carcinogens*	*Salmonella* mutagenicity	Rodent bone marrow chromosomal aberrations or micronuclei
Organic compounds		
Aflatoxins	+	+
4-Aminobiphenyl	+	+
Analgesics containing phenacetin	+	+
Azathioprine	+	+
Benzene		+
Benzidine	+	+
Betel quid and tobacco	+	+
Bis(chloromethyl) ether	+	(+)
Chlorambucil	+	+
Chlornaphazine	+	+
Cyclophosphamide	+	+
Melphalan	+	+
Mustard gas	+	ND(+)
Myleran	+	+
2-Naphthylamine	+	+
Tobacco, smokeless	+	+
Tobacco, smoke	+	ND(+)
Treosulphan	+	+
Vinyl chloride	+	+
Soot, tars, and oils		
Coal tar pitch	+	ND
Coal tar	+	ND
Mineral oil (untreated and mildly treated)	+	ND
Soot	+	ND(+)
Metals		
Arsenic compounds		+
Chromium compounds (hexavalent)	+	+
Nickel and nickel compounds		ND

+ = positive response; (+) = predicted positive response; ND = not tested so far.
* The carcinogenic hormones (essentially natural and synthetic estrogens) and fibers (asbestos, erionite, and talc containing asbestiform fiber) are not included; the members of these groups tested so far gave negative or inconclusive results in the *Salmonella* mutagenicity and rodent bone marrow clastogenicity assays.

known. However, these considerations also suggest that although positive results in the short-term assays for genotoxicity indicate carcinogenic potential and may give reason to interrupt further development of a compound, negative results cannot be taken as a guarantee of the absence of carcinogenic activity in vivo, but rather as a green light to proceed to the lifelong rodent bioassay.

4.10. Evaluation of Toxic Effects on the Immune System

In the last twenty years experimental and epidemiological evidence has accumulated that the mammalian immune system may be altered (i.e. suppressed or induced) by a wide range of environmental chemicals and drugs. Well-known examples are the polychlorinated and polybrominated biphenyls; dibenzo-*p*-dioxins; benzene; isocyanates; metals such as chromium, lead, and nickel; and organometallics such as di-*n*-octyltin chloride and tri-*n*-butyltin oxide.

Immunotoxicity is the ability of a substance to adversely affect the immune system: the immune response of affected individuals is altered. Immunotoxic responses may occur when the immune system is the target of the chemical insult; this in turn can result in either immunosuppression and a subsequent decreased resistance to infection and certain forms of neoplasia, or immune disregulation, which exacerbates allergy or autoimmunity. Alternatively, toxicity may arise when the immune system responds to an antigenic specificity of the chemical as part of a specific immune response (i.e., allergy or autoimmunity). Changes in immunological parameters may also be a secondary response to stress resulting from effects on other organ systems. Therefore, it must be recognized that in principle all chemical substances may be able to influence parameters of the immune system if administered at sufficiently high dosages. However, an immunotoxic effect should only be discounted when a thorough investigation has been performed. Although the immune system is considered as a target organ with regard to systemic toxicology, it consists of several different organ systems. A very large number of different cell types, present in practically all tissues and compartments of the human or animal body, participate in the immune response. Due to the complexity, it is not possible to describe the immune system in the context of the present chapter. Only a very brief description will be given to help the understanding of the current practical approaches of the evaluation of adverse effects on immune function induced by chemicals (see also → Immunotherapy and Vaccines, Chap. 1).

The overall immune system can be categorized in two major subsystems: the humoral and the cell-mediated subsystems. The effects of the humoral system are mediated by B lymphocytes producing antibodies that react with antigenic (usually foreign) material (i.e., antibodies attack bacteria and viruses before they can enter the host cell). The cell-mediated system involves primarily the mobilization of phagocytic leucocytes (macrophages) to ingest foreign organisms such as bacteria and the activation of T lymphocytes.

The two systems do not function independently in different situations; rather, they interact by complex feedback mechanisms that are only partly understood. One of the main properties of the immune system is the rapid production of a large number of cells capable to react with a specific antigen when this antigen is presented (again) to the organism. This important property is based on the presence of a wide variety of memory cells that were specifically adapted to the antigen at the time of initial contact.

Immunocompetent cells are required for host resistance, and thus exposure to immunotoxicants can result in increased susceptibility to any type of toxicity and disease including cancer.

The evaluation of adverse effects on the immune system can be carried out in two tiers [183, 184]. The first tier evaluates immune-related parameters (haematology, blood chemistry, organ weights and histopathology) that are included in revised standard testing protocols of repeated-dose (28 and 90 d) toxicity studies (Table 35).

Compounds showing some immunotoxic properties in this first tier and also chemicals suspected of having immunotoxic effects based on information from prior studies or structure–activity relationships are further evaluated by functional assays that assess competence of immune cells (Table 36) For a detailed description of the experimental procedures see [196].

Table 35. Immunological parameters that can be included in repeated-dose toxicity studies in rats for compounds for which no prior immunotoxic potential has been identified (tier I)

Parameters assessed	Tests
Hematology and blood chemistry	differential white blood cell counts
	bone marrow cellularity
	albumin : globulin ratio
	serum immunoglobulin classes
Organ weights	thymus, spleen, lymph nodes
Histopathology	thymus, spleen, lymph nodes, bone marrow, Peyer's patches (if oral administration)
	bronchus-associated lymphoid tissue (in case of pulmonary administration)

Table 36. Selected functional assays to assess immunotoxicity of compounds for which some immunotoxic properties have been implied in tier I evaluation (see Table 35) or in prior studies (tier II)

Parameter assessed	Tests
Cell-mediated immunity	mixed leukocyte response to antigenic determinants; induction of cell proliferation by the T-lymphocyte mitogen concanavalin A; T-lymphocyte cytotoxicity; delayed hypersensitivity response (DHR) to keyhole limpet hemocyanin (in contrast to the first three assays, that are carried out in cultured cells in vitro, the DHR is induced in vivo)
Antibody-mediated immunity	antibody plaque-forming cell response; serum antibody titer after exposure to specific antigens determined by enzyme-linked immunosorbent assay (ELISA)
Natural and induced host resistance	natural killer cell cytotoxicity; macrophage-mediated phagocytosis and intracellular killing; assessment of macrophage and T-lymphocyte competence by inoculation of *Listeria monocytogenes*; host resistance to melanoma cells

4.11. Toxicological Evaluation of the Nervous System

Neurotoxicity is the induction by a chemical of adverse effects in the central or peripheral nervous system, or in sensory organs. It is useful for the purpose of hazard and risk assessment to differentiate effects specific to sensory organs from other effects which lie within the nervous system. A substance is considered neurotoxic if it induces a reproducible lesion in the nervous system or a reproducible pattern of neural dysfunction.

The identification and characterization of neurotoxic properties of chemicals is one of the essential goals of every toxicity screening programm. Neurotoxicity has traditionally been associated with structural pathological modification of nervous system constituents (i.e. neurons, glial cells, and endothelial cells). Hence, the most important tool to assess neurotoxic effects in the past has been the histopathological examination of the nervous system of animals acutely or chronically exposed to xenobiotics. Over the last ten or twenty years, however, several reasons have called for the development and use of functional tests in neurotoxicity screening. First, detailed histopathological analysis of the nervous system is very time-consuming and requires experienced neuropathologists. Second, many chemical compounds disturb the nervous system without causing identifiable structural lesions. This clear need for functional tests to assess neurotoxicity has led to the development of a functional observational battery and an automated test to detect locomotor activity in rats based on methods that have been used by neuropharmacologists for many decades for the evaluation of psychoactive neurologic and autonomic pharmaceutical compounds.

4.11.1. Functional Observational Battery

The functional observational battery can be carried out both in acute and repeated-dose toxicity studies. The sequence of tests is usually arranged to progress from the least to the most interactive with the animal. The assessment begins with home cage observations followed by measurement made while handling the animals and assessment of activities in the open field. Assessment of reflexes, as well as physiologic and neuromuscular parameters is carried out at the end of the examination. Specific parameters assessed at these different stages are summarized in Table 37, and more detailed descriptions of some important tests are given below (reviews: [197–199]).

Catalepsy. Catelpsy can be measured by placing the rat on four corks (35 mm high, 40 mm in diameter, 100 mm between fore- and hindfeet, 60 mm between right and left feet).

Duration of immobility at this position is measured for a period up to 60 s. For the assessment of catalepsy, a number of experimental modifications exist (i.e. placing the rats on a horizontal bar 12 cm above the ground). However, the four-corks procedure allows the best distinction between cataleptic and heavily sedated animals.

Table 37. General parts of the functional observational battery to assess neurotoxicity and specific examples of parameters recorded

A.	home cage observations	posture
		palpebral closure (eyelids wide open to completely shut)
		convulsions (clonic, tonic)
		biting
B.	observations while handling the animal	ease of removal from cage and of handling in hand, lacrimation, salivation, piloerection, fur appearance
C.	open-field activity (observations over 3-min period)	time to first step
		number of rears (supported and unsupported)
		number of urine pools and defacations
		mobility and gait
		tremors, ataxic gait, convulsions
D.	reflexes	approach response (e.g., to a pencil)
		touch response
		click response
		tail response
		pupil and eye blink response
		forelimb and hindlimb extension
		righting reflex: hold rat in supine position, drop from approximately 30 cm and note ease of landing
E	physiologic observations	catalepsy
		body temperature
		body weight
F	neuromuscular observations	rotarod performance
		grip strength
		hindlimb extension strength
		hindlimb foot splay

Grip Strength. The rat is allowed to grip a triangular ring with its forepaws and is pulled back along a platform until its grip is broken. As the pulling back continues, the animal's hindpaws reach a T-shaped rear limb grip bar, which it is allowed to grasp and then forced to release by continued pulling. Special devices are used to measure the maximum strain required to break forelimb and hindlimb grip.

Rotarod Performance (Rotating-Rod Test). This test requires preliminary training of the animals to walk on a rotating rod (7 cm in diameter, 5–12 rpm rotation rate). During the training period, the containers underneath the rod are filled with water to prevent the rats from jumping off. For testing, the time each rat remains on the rotarod is measured up to 2 or 3 min. Due to its objectivity and reproducibility this test is one of the most popular for the evaluation of adverse effects to the nervous system.

Hindlimb Foot Splay. The hind feet are painted, animals are dropped from a horizontal position 30 cm above a table onto paper and the distance between the middle of the ink spots is measured.

4.11.2. Locomotor Activity

Locomotor activity is not automatically part of the screening battery for neurotoxicity, the decision to carry out the test being rather made individually based on preliminary observations. Locomotor activity is assessed in special test chambers using a photocell detection procedure. Animal movement inside the chamber interrupts the photobeams and is translated into activity counts. Data on animal activity are usually recorded over three 5 min intervals.

In addition, a number of further methods to investigate neurotoxicity are available and standardized approach for an evaluation may not be given, but need to be decided on a case-by-case basis (Table 38).

Table 38. Methods for investigation of neurotoxicity

Effect	Methods available
Morphological changes	Neuropathology
	Gross anatomical techniques
	Immunochemistry
	Special strains
Physiological changes	Electrophysiology (e.g. nerve conduction velocity, NCV)
	Electroencephalogram (EEG, evoked potentials)
Behavioral changes	Functional observations
	Sensory function tests
	Motor function tests (e.g., locomotor activity)
	Cognitive function tests
Biochemical changes	Neurotransmitter analyses
	Enzyme/protein activity
	Measures of cell integrity

A major problem in neurotoxicity screening is that the general behaviour of rats is not stable and uniform, even when they are kept under strictly controlled conditions and not exposed to neurotoxic compounds. Therefore, many authors suggest to carry out the tests without knowledge of the treatment protocols. Furthermore, since many observations are subjective, it is important to use experienced and well trained scientists and technicians in the evaluation.

4.12. Effects on the Endocrine System

Although endocrine disruption is often regarded as a specific end point in toxicity testing, it is just a mechanism by which a chemical may induce adverse effects. Many endocrine-dependent toxicities will be detected in the course of the already available methods for toxicity testing. However, a number of specific tests for endocrine effects of chemicals are under discussion for introduction into toxicity testing.

The endocrine system consists of a number of glands such as the thyroid, gonads, and the adrenals, and the hormones they produce such as thyroxine, oestrogen, testosterone and adrenaline. These hormones may influence development, growth, reproduction and behaviour of animals and humans.

Endocrine disrupters are defined as:

- Chemicals that have properties that might be expected to lead to endocrine disruption in an intact organism, its progeny, or (sub)populations
- Chemicals that alter functions(s) of the endocrine system and consequently may cause adverse health effects in an intact organism or its progeny

Endocrine disrupters may to interfere with the endocrine system by several mechanisms:

- By mimicking the action of a naturally produced hormone such as oestrogen or testosterone and thereby inducing similar chemical reactions in the body
- By blocking the hormone receptors in cells and thus preventing the action of normal hormones;
- By affecting the synthesis, transport, metabolism, and excretion of hormones and thus altering their concentrations.

A variety of test systems to characterize the potential of chemicals for endocrine disruption, ranging from receptor-binding assays to complex in vivo measurements, have been proposed, but a specific testing approach has not yet been agreed.

Some testing guidelines (e.g., the 28-d study guideline) detect effects on endocrine function, but there are no test strategies/methods available which can detect all possible effects that may be linked to the endocrine disruption mechanism.

5. Evaluation of Toxic Effects

One of the major environmental and occupational issues of concern to both scientists and administrators is the control of potential health hazards to humans due to the production, use, and disposal of chemicals. The concern arises from the increasing numbers of chemicals in production and use and the increasing numbers of chemicals demonstrated to exert toxic effects in one or several of the sensitive toxicity testing systems available. This situation has afforded growing legislative control of the production and application of chemicals to ensure adequate protection of human health. Control measures based on the recognition of potential adverse health effects may limit the presence of hazardous chemicals in the environment or regulate the use of hazardous chemicals, thus reducing the potential health risks to humans (Table 39).

Table 39. Possible measures to reduce human exposure to hazardous chemicals

Application or exposure to chemical in question	Measures to reduce exposure
Industrial chemicals	reduction or cessation of application; protective measures in the workplace; alternative chemicals with lower hazard
Pharmaceuticals	cost – benefit analysis
Alcohol, smoking, drugs of abuse	education
Environmental chemicals	quantitation of exposure, strategies for avoidance or reduction of environmental pollution

The assessment of potential human health risks resulting from the exposure to chemicals

provides the basis for appropriate regulatory and control measures. The health risk assessment determines whether a xenobiotic may cause adverse health effects, at what level and frequency of exposure, and the probability that adverse health effects will occur. The term "risk assessment" is increasingly used in the context of potentially toxic chemicals. Scientific risk assessment considers the available data on the toxicology of a specific chemical when judging which agents potentially pose a significant risk to the human population. Toxicology focuses on the identification and quantitation of potential hazards by using animal studies as surrogates for humans. Permissible exposure levels for humans are derived from the results of the animal studies by using margins of safety or defining "acceptable" incidences of adverse health effects in exposed humans [185].

Health risk assessment and its use in regulatory decisions have recently generated intense controversy. The debate over risk assessment is politically and emotionally charged, and creates an adverse atmosphere heightened by the extraordinary sums of money at stake. Industry complains that the costs of complying with possible overregulation based on inappropriate risk assessments may be excessive; moreover, lawsuits on potential environmentally caused diseases, especially in the United States, involve huge sums of money. On the other hand, environmentalists claim that risk assessment practices and policies do not adequately protect human health; moreover, health care costs for the treatment of environmentally caused diseases may also be very high. These considerations have led to an intensive rethinking of the health risk assessment process and have increased the awareness that in many cases, the scientific foundation for risk assessment is weak. This rethinking led to the conclusion that resolution of the controversies by the development of effective prevention strategies and rational priority setting may be achieved only by strengthening the scientific background and available data by research and by developing better methods to estimate risks due to chemical exposures [185–187].

Before considering the practice of health risk assessment, several terms frequently used and misused in risk assessment and its perception should be clarified. In discussions on health effects of potentially toxic chemicals, the terms "hazard" and "risk" are often used with an identical meaning, although they are clearly different. Hazard defines the intrinsic toxicity of a chemical and is not identical to risk. Risk is the estimated or measured probability of injury or death resulting from exposure to a specific chemical. Risk may be described either in semiquantitative terms such as high or low risk or in quantitative terms such as one person experiencing an adverse effect per 10 000 persons exposed. Risk may also be described in absolute terms (probability of adverse effects due to a specific chemical exposure) or in comparative terms by comparing the probability of adverse effects between a population exposed to an agent and an unexposed population.

The health risks due to the contact with potentially toxic chemicals are dependent on the conditions of exposure, since not only the intrinsic toxicity of a chemical determines the magnitude ot the adverse effect but also the dose. As noted in Chapter 1, the magnitude of the toxic effects is the product of the intrinsic toxicity of a chemical multiplied by the dose taken up by exposed animals or humans; thus, all toxic effects are dose-dependent and even very toxic chemicals may not cause toxic effects when the dose is low. If the dose is zero, despite a very high intrinsic toxicity of a specific chemical, the toxic effect and the risk of adverse health effects will be zero. On the other hand, chemicals with low intrinsic toxicity may induce toxic effects when the dose is high and may thus pose a significant risk. In toxicological terms, risk is therefore the product of the intrinsic toxicity of a chemical and the exposure characteristics.

5.1. Acceptable risk, Comparison of Risks, and Establishing Acceptable Levels of Risk

In earlier phases of risk assessment, the basic belief was that few chemicals are toxic and all of these toxic chemicals are derived from synthetic processes. To achieve a zero risk, chemical exposure must be reduced below a threshold level, under which it causes absolutely no risk. However, where such a threshold cannot be demonstrated, one must assume that a finite risk may occur at any exposure level, consequently, absolute control of risk is possible only if the source

of exposure is eliminated altogether. These considerations resulted in the zero-risk concept. The Delaney Clause of the Federal Food and Drug Act in the United States is an example of a zero-risk approach in the regulation of food additives. This law states that no xenobiotic whose carcinogenic potency in animals has been demonstrated may be used as a food additive.

However, the more widespread testing of chemicals for toxicity, the increased sensitivity of analytical instruments to determine chemicals in the environment and at the workplace, and the developments in the science of toxicology put the basic assumption of the zero risk concept – that is, only synthetic chemicals are toxic – into question.

However, the more widespread testing of chemicals for toxicity, the increased sensitivity of analytical instruments to detect chemicals in the environment and the workplace, and developments in the science of toxicology put the basic assumption of the zero-risk concept – that only synthetic chemicals are toxic – in question. These developments led to the recognition that zero risk was unachievable and, perhaps, unnecessary for the regulation of chemicals. The observation was based mainly on a few facts: (1) all chemicals, both of synthetic and natural origin, are toxic under specific exposure conditions; (2) most of the hazardous chemicals routinely encountered by humans are of natural rather than synthetic origin; (3) most of the exposure to hazardous synthetic chemicals cannot be avoided entirely or be eliminated from the environment without changing profoundly the way of life in many countries; and (4) in the case of cancer risk assessment, DNA damage and mutations, assumed to be of major significance in the process of carcinogenesis, occur spontaneously, albeit at a low rate. Examples of endogenously occurring DNA damage are hydrolytic deamination, depurination, oxidative modification, and endogenously formed DNA adducts. Well-known examples of hazardous synthetic chemicals are benzene, which is present in the environment as a result of its emission from motor vehicles, cigarette smoking, and other sources, or 2,3,7,8-tetrachlorodibenzodioxin formed in forest fires. The effect of naturally occurring chemicals and chemical exposure due to life-style factors is best exemplified by the estimated contribution of different factors to the incidence of avoidable cancers in humans (Fig. 68).

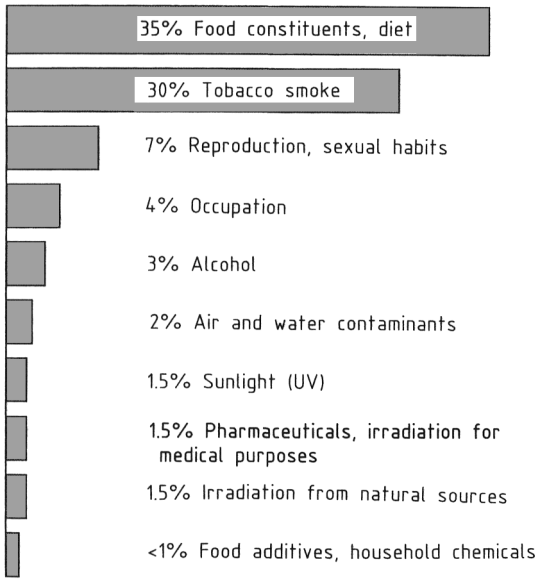


Figure 68. Contribution of chemical exposure and life-style factors to the incidence of avoidable cancer in human [200]

According to the large epidemiological study of Doll and Peto (1981), natural chemicals in diet and chemicals inhaled by cigarette smoking are the major causative agents in human cancer. Occupational and environmental exposure to synthetic chemicals constitutes only a minor causative factor.

Given these facts, the acceptable risk concept was developed as an alternative. The acceptable risk concept realizes that it is not possible to eliminate all potential health risks associated with chemical exposure due to the life style. According to the concept of acceptable risk, safety – the reciprocal of risk – is no longer an absolute term but is redefined as a condition of certain, but very low and thus acceptable, risk. This conceptual change improves the ability to deal with potentially very low risks identified by the increased sensitivity of analytical instrumentation and with increasingly sensitive scientific methods to detect potential adverse effects of xenobiotics. The concept of acceptable risk also permits the definition of limits for the exposure to toxic chemicals that can be considered to have a

negligible impact on the incidence of adverse effects in an exposed population. Risk assessment is therefore unavoidable and must implicitly or explicitly involve a balance of risk and benefit [185–189]. Some of the main factors considered in establishing acceptable risk levels for exposure to a chemical follow:

Beneficial aspects
- Economic growth
- Employment
- Increased standard of living
- Increased quality of life
- Taxes generated

Detrimental aspects
- Decreased quality of life
- Health effects
- Lawsuits
- Loss of environmental resources
- Loss of work
- Medical expenses

5.2. The Risk Assessment Process

Several individual elements make up the risk assessment process. In the first step, the potential adverse health effects of a chemical, a mixture of chemicals or a specific technical process are evaluated by the application of toxicity tests (for details, see Chap. 4) and, if the chemical is already in widespread use and humans are exposed, by considering the data from epidemiological studies. The second step in risk assessment determines the dose–response for the observed adverse effects. In parallel, the exposure of humans to this xenobiotic is quantitated by analytical procedures or, if the chemical is not yet in widespread use, by the estimation of likely exposure scenarios. In the third step, the results obtained in the toxicity studies are extrapolated. This involves an extrapolation both from adverse effects seen in experimental animals to humans, and often an extrapolation from the effects seen after high doses in animals to the much lower doses humans usually encounter. The last step of risk assessment, risk characterization, involves the combination of steps one to three to judge the existence and magnitude of the public health problem and characterizes the uncertainties inherent in the risk assessment process

5.2.1. Hazard Identification Techniques

Hazard identification is the step in which the adverse effects of the xenobiotic are determined. Evaluation of both acute and chronic toxicity is performed by using animals as experimental models for humans. The use of animals as surrogate for humans is based on the following assumptions: (1) xenobiotics with a likely adverse effect in humans will manifest some degree of toxicity in other living systems when the dose is sufficiently high; (2) if a sufficient number of animal species are dosed with the xenobiotic, at least one should exhibit a similar pattern of biotransformation and toxicokinetics to that seen in humans; and (3) if a sufficient number of different animal species are dosed with the xenobiotic, at least one is likely to exhibit the toxic responses and clinical symptoms occurring in humans.

Typical end points in studies aimed at hazard identification in intact animals include mortality, reproductive and developmental effects, target organ toxicity, and cancer. Hazard identification studies at present also include the determination of a range of biochemical end points related to specific toxic effects such as toxicokinetics, routes and extent of biotransformation, structure of reactive intermediates, and binding of reactive intermediates to cellular macromolecules. In addition, many short-term tests for specific toxic effects such as DNA damage, mutagenicity, or clastogenicity are increasingly included in hazard identification procedures.

The acute toxicity of xenobiotics is evaluated by a number of procedures from which the LD_{50} may be calculated. With the more recently recommended fixed-dose method information on target organs affected and types of toxic effects may also be obtained. Repeated-dose toxicity studies last between two weeks (subacute toxicity studies) and 6–24 months (chronic toxicity studies), the lifespan of the animals, including post-mortem examination, histopathology, clinical chemistry, and hematology at termination and at specified time points during the study. From the chronic toxicity studies, the lowest observed effect level (LOEL), also referred to as the lowest observed adverse-effect level (LOAEL), and the no observed effect level (NOEL) are obtained for noncancer endpoints. The NOEL is

the highest does administered that does not induce observable toxic effects. The NOEL may not be identical to the no-effect level if insensitive methods are applied or the wrong end point is chosen. Moreover, the value obtained for the NOEL will depend on the number of animals used in the study and the spacing of the applied doses.

Short-Term Tests for Specific Toxic Effects *In Vivo* and *In Vitro*. A variety of short-term tests has been developed for the detection and quantification of toxic effects. Most of the more established and well-evaluated test systems are designed to evaluate the genotoxic activities of xenobiotics and employ well-defined genetic changes (DNA damage, gene mutations, chromosome defects, cell transformation) in prokaryotes, lower eukaryotes, and mammalian cells. Knowledge that a genotoxic chemical is active in vivo in the target organ of carcinogenesis enhances confidence that the genotoxicity of the chemical is important in the process of cancer induction by that chemical. Before any short-term test can be used with confidence to assess potential toxic effects, its validity should be thoroughly evaluated and its major drawbacks should be explicitly noted. Moreover, it should be kept in mind that most available short-term tests are well designed to give qualitative information and their major use for risk assessment purposes is therefore the confirmation or exclusion of a specific toxic response. The magnitude of toxic response in intact animals or humans depends on both toxicokinetics and toxicodynamics. The toxicokinetic phase of the toxic response is not considered in most of the in vitro short-term tests. Therefore, short-term tests in vitro may be used for hazard identification only in combination with studies on adverse effects in animals and studies on the toxicokinetics of a xenobiotic.

Biotransformation and Toxicokinetics. Studies on the extent of biotransformation including structural identification of the metabolites formed from the xenobiotic both in intact animals and in appropriate in vitro systems such as organ homogenates or fractions with enzymatic activity also contribute to hazard identification [190]. The structures of metabolites formed and the presence and extent of covalent binding of metabolites to macromolecules such as protein and DNA indicate the formation of electrophilic metabolites and thus a potential hazard.

For example, the structure of excreted mercapturic acids may give information on the structure and reactivity of the electrophilic metabolite and sites of cellular interactions [191]. Information on the rate of absorption and elimination may indicate a possible accumulation of the xenobiotic in humans, with the consequence of potential adverse effects. These studies should be performed on at least two animal species in vivo. For in vitro studies, human tissue samples should be included to confirm that biotransformation reactions are identical to those, observed in animals occur in humans. Different mechanisms of toxicity may operate at different dose levels; in these instances, toxicokinetic data may help in understanding dose-dependent mechanistic differences. The toxicokinetics of a xenobiotic in humans may be extrapolated by physiologically based pharmacokinetic models from the results obtained in experimental animals. Information on potential pathways of biotransformation may also be made by computerized structure analysis of the xenobiotics with specific computer programs [147, 192]. These are designed for predicting routes and rates of biotransformation based on the presence of functional groups in the molecule; however, at present, the available programs are far from perfect, and they should only be used in conjunction with expertise and as a basis for experimental planning.

Structure – Activity Relationships and Chemical Structure Analysis. Predictive data on the potential of xenobiotics, mainly organic chemicals, to induce adverse effects may be derived from relationships between chemical structure (physicochemical properties, presence of functional groups, atomic configuration) and biological activity, termed structure–activity relationships.

Two approaches to hazard prediction utilize structure–activity relationships. The first is essentially a qualitative approach and involves the comparison of the structure of the xenobiotic with that of other compounds already known to cause specific toxic effects. The comparison with known structures and the knowledge of

biotransformation reactions and mechanisms of toxicity permits the identification of toxophores in the structure of xenobiotics. Toxophores are functional groups present in the molecule which are likely converted to toxic metabolites or posess chemical reactivity related to mechanisms of toxicity. Examples of toxophores are olefinic moieties, which may be oxidized to epoxides, and terminal carbon atoms in aliphatics bearing two halogen atoms, which may be oxidized to acyl halides. The scope and usefulness of structure–activity relationships depend on the availability of a sufficiently large database on the toxic effects of chemicals with common subgroups and structures [147], [192]. The main limitation of this approach is the qualitative nature of the resulting estimate of potential toxicity. The second approach involves quantitative structure–activity relationships and relies on the computer-aided analysis of databases on toxic effects of chemicals. A basic feature of the applied techniques is the use of pattern-recognition schemes or substituent weighting factors coupled with regression analysis. Some useful predictions have been made with these techniques, but due to the complex nature of toxic effects and the multitude of factors governing the toxic response, there are still severe limitations to the applicability of quantitative structure–activity relationships for predicting toxicity profiles.

Clinical and Epidemiological Studies. In principle, the best evidence for the toxic effects of a chemical in humans is derived from clinical and epidemiological studies. These studies assess effects in the species of interest for risk assessment and at relevant concentrations. Potentially confounding extrapolations from high to low dose and from animals to humans are not required. However, several weaknesses limit the applicability of these studies to hazard identification. In most cases, reliable exposure data are lacking, consequently, dose–response relationships cannot be established. In addition, the sensitivity of epidemiological studies to detect health problems is comparatively low. Unless the toxic effect of a particular xenobiotic is very unusual in control (unexposed) groups, it may pass unnoticed in a normal survey. Examples are the identification of asbestos exposure as a cause of mesothelioma or vinyl chloride as a cause of hemangiosarcoma, very rare forms of cancer in humans not exposed to asbestos or vinyl chloride, respectively. Moreover, the results of epidemiological studies, especially on cancer risk, may reflect the risk associated with exposure to chemicals decades ago because of the long latency period. Other limitations are confounding variables such as smoking and concomitant exposure to other xenobiotics, which often impair the interpretation of carcinogenicity data in humans. Thus, evidence based on epidemiological observations has identified only a limited number of chemicals as human carcinogens; many of the identified compounds are used in cancer chemotherapy and have the intrinsic property of genotoxicity (Table 40).

Table 40. Examples of established human carcinogens based on epidemiological observations

Chemical or agent	Site of tumor formation
Aflatoxin	liver
Alcoholic drinks	mouth, esophagus
4-Aminobiphenyl	bladder
Benzidine	bladder
2-Naphthylamine	bladder
Arsenic	skin, lung
Asbestos	lung, pleura, peritoneum
Azathioprine	reticuloendothelial system
Benzene	bone marrow
Bis(chloromethyl) ether	lung
Cadmium	prostate
Chlorambucil	bone marrow
Chlornaphazine	bladder
Chromium	lung
Cyclophosphamide	bladder
Bis(2-chloroethyl) sulfide	larynx, lung
Nickel compounds	nasal cavity, lung
Estrogens	endometrium, vagina
Phenacetin	kidney and lower urinary tract
Polycyclic aromatic hydrocarbons	skin, scrotum, lung
Steroid hormones	liver
Tobacco	mouth, pharynx, larynx, esophagus, lung, bladder
Vinyl chloride	liver

5.2.2. Determination of Exposure

The quantification of exposure, both in individuals and in populations, is a prerequisite for the quantification of risk. Reliable data on exposure are needed to assess the adverse effects of the xenobiotic and to recognize specific risk factors such as occupation, life style, and social status. The dimensions of exposure include intensity, frequency, route, and duration; in addition, the nature, size, and makeup of the exposed population should be characterized. The assessment

of exposure is a difficult and complex task, and is often neglected. Typically, estimations and field measurements are required. The estimation of human exposure to a particular xenobiotic involves an initial estimation of the possible sources of the chemical and the possibilities for exposure. A good inventory of sources may provide important information on critical pathways of exposure, populations at particular risk, and the levels of exposure.

In many cases, the duration and level of exposure, especially after chronic contact, may only be estimated from ambient levels of the xenobiotic in the environment, and estimations may thus be crude; owing to the large numbers of potentially exposed persons, only in special situations (e.g., occupational exposure, after disasters), will exposure data, including determination of the internal dose, be available.

Specific procedures to detect exposure to a certain xenobiotic and procedures to estimate exposure by determining biological effects may be used. These include:

- Direct measurement of the chemical in environmental samples such as water, air, and soil.
- Measurement of the chemical, its metabolites or products of the interaction of the chemical or its biotransformation products with cellular macromolecules (protein and/or DNA) in body fluids and tissues (biomonitoring).

Biological end points in exposure assessment may be:

- Assessment of biochemical indicators for specific adverse effects known to be caused by the xenobiotic, e.g., inhibition of specific enzymes such as cholinesterase activity in persons exposed to organophosphate pesticides.
- Observation of pathological evidence of exposure such as cytogenetic changes in lymphocytes from workers exposed to chromosome-damaging chemicals at the workplace. However, the use of biological end points for exposure assessment lacks the resolving power to discriminate between endogenous changes and the effects of xenobiotics, and therefore chemical specific indices of exposure should be favored for quantitation of exposure [178, 193].

Due to the time-consuming and cost- and labor-intensive procedures required, data on exposure to xenobiotics are usually limited. Difficulties in identifying concomitant exposures, interactions with other xenobiotics or activities, special risk groups such as the very old or very young and pregnant women, and patterns of exposure result in a high degree of uncertainty in exposure assessment in human populations.

A stepwise approach to exposure assessment using the following hierarchy can be applied. The most reliable exposure assessments are based on measured data, including the quantification of key exposure determinants. When these are not available, appropriate surrogate data may be used. Modeling may also be used in the absence of useful data, but the limitations of the modeling approach should be clearly stated.

5.2.3. Dose-Response Relationships

The establishment of the dose–response relationship for adverse effects in animals is the decisive step in risk assessment. This step quantifies the relationship between received or administered dose and biological response and may be performed on an individual or population basis. Dose–response assessment includes exposure intensity and duration and factors modifying toxic response such as sex, age, health status, and route of administration. Since the dose–response assessment can only rarely be performed in humans, extrapolation from data obtained in animals to humans is usually required. Moreover, many animal experiments, particularly carcinogenicity bioassays, are performed with high doses to increase the sensitivity of the assay. Therefore, besides species extrapolation, an extrapolation from effects seen after high doses in animals to the low doses usually encountered by humans is necessary. This extrapolation step from high, sometimes toxic doses in animals to low doses in humans is controversial.

The extrapolations form high to low dose are performed differently depending on the type of toxic response elicited by the xenobiotic. As explained in Chapter 1, toxic effects of a chemical may be caused by both reversible and irreversible interactions of the xenobiotic or its

metabolite(s) with macromolecules in the organism. Many acute toxic responses such as carbon monoxide poisoning are based on reversible interactions and are associated with thresholds. Threshold doses are doses below which the probability of a response is zero. The biological basis for thresholds is well founded and may be demonstrated on a mechanistic basis. On the other hand, many chronic toxic responses, particulary chemical carcinogenesis, are often considered nonthreshold effects. Since a negative can never be proven, the absence of thresholds cannot be demonstrated by experiments and is based on consideration of the mechanisms of chemical carcinogenesis. Different approaches have thus been developed in establishing acceptable levels of exposure to threshold and nonthreshold responses (see below).

5.2.4. Risk Characterization

The hazard identification, the exposure assessment, and the dose–response assessment merge into risk characterisation. Risk characterisation estimates the incidence of expected adverse health effects in exposed populations. As noted in Section 5.2.1, risk characterization and the establishment of acceptable exposure levels are handled differently for carcinogenic and noncarcinogenic xenobiotics. For chemicals that cause adverse effects by mechanisms with thresholds, the safety factor approach was developed. For nonthreshold responses such as cancer, both quantitative and qualitative risk assessment procedures are used.

5.2.4.1. The Safety-Factor Methodology

When the safety assessment of a chemical is based on animal toxicity testing, a different evaluation of the data is required compared to safety evaluations relying primarily on human observations. Since animal experiments to determine health hazards of chemicals were widely used in the 1940s and human health risks had to be extrapolated from these data, the idea of a safety factor, which was to be applied to the results from animal studies, was developed. The safety factor approach was first proposed in the 1950s by Arnold Lehmann of the FDA as a protective measure for human health and is intended to compensate for uncertainties in the extrapolations of animal data to humans. This concept was applied to the determination of acceptable daily intakes (ADIs) by the World Health Organization from the 1960s onward. The ADI was defined as "the daily intake of a chemical which, during an entire lifetime, appears to be without appreciable risk on the basis of all known facts at that time."

The safety factor, or as it is sometimes called, the uncertainty factor, has been introduced to consider both interspecies and interindividual differences in response to potential toxic effects of the chemical under consideration. The major purpose of the safety factor is the protection of human health by establishing safe exposure levels; the exposure levels defined do not mean that exposure above these levels will result in adverse effects. Despite several limitations and criticisms, the safety factor approach has been used for many years in Western Europe and the United States, and has proven useful and reliable. It was thus adopted internationally as the standard procedure for assessing the ADI.

This risk assessement approach is based on the establishment of a point of departure for the required extrapolation, which may be an NOEL or NOAEL or a benchmark dose (e.g. ED_{05}) in animal studies on chronic toxicity, and then defining permissible human exposures by the application of a safety factor. In this context, the NOEL is defined as the lowest dose of the xenobiotic in an animal experiment which produced no detectable effect in the most sensitive animal species treated. Once a NOEL has been determined, a safety factor for human exposure is introduced and, often, a permissible level of exposure of one hundredth of the NOEL in animals is defined for humans. The 100-fold safety factor is justified on the basis of a 10-fold difference to reflect an interspecies difference in susceptibility and a 10-fold difference to reflect possible interindividual variations in susceptibility in humans [188]. The acceptable daily intake is then obtained by dividing the NOEL from the study by the safety factor.

The safety-factor approach assumes that toxic effects exerted by the chemical exhibit a dose–response curve with a threshold, that the results of the toxicity studies in animals are relevant to humans, and that extrapolation of the dose is reliable.

A similar approach is the margin-of-safety (MOS) approach which determines by how much the derived NOAEL exceeds the determined or estimated exposure. The size of the MOS (< 100 or > 100) determines the extent of concern of a specific exposure.

As noted above, safety factors of 100 are frequently applied. However, when reliable data for adverse effects of the chemicals in humans are available, a safety factor of only 10 may be applied. On the other hand, a safety factor of more than 100 is appropriate when no or only limited data on the toxicity of the chemical in animals are available. Note that the safety factor is not based on scientific evidence. Additional data on the mechanisms of toxic effects in animals and on the toxicokinetics and biotransformation of the xenobiotic in animals and humans are required and add scientific credibility to the proposed safety factors. Therefore, the choice of a safety factor should consider, in addition to the NOEL, a series of further qualitative parameters:

Evaluation of animal toxicity studies
- Number of studies and effects observed
- Type of toxic effects
- Time course for toxic effects
- Tumorigenicity

Evaluation of biochemical end points
- Biotransformation and toxicokinetics
- Mechanism of action, covalent binding to macromolecules
- Short-term test for genotoxicity and other nonthreshold effects

Evaluation of species differences
- Interspecies variations in biotransformation and toxicokinetics
- Influence on anatomical and physiological differences of toxic effects between species

Application of the most appropiate safety factor requires careful analysis of the data available on the toxic effects for every chemical, on a case-by-case basis. Consideration should be given to the quality and completeness of the data and the number and spread of the dose levels used. Besides the derived NOEL, all further information obtained in the long-term animal toxicity studies is valuable for characterizing toxic effects and determining safety factors. The type of toxic effect and the shape of the dose–response curve should also be taken into account in setting the size of the safety factor.

Also important are considerations of the ability to extrapolate from toxic effects seen in animals to human exposure scenarios and the results of human epidemiology studies, when available. Because of the complexity of information required, no universally accepted guidelines can be developed for determination of the precise magnitude of safety factors; therefore, expert judgment on an individual basis is a major factor contributing to the size of safety factors.

The safety factor approach has several problems. In experiments to determine the NOEL in animals, the experimental group size and the spacing of dose levels are major determinants of the numerical value of the NOEL obtained; the smaller the size of the individual dose groups and the larger the spacing of doses, the less likely is it that an effect will be observed. This phenomenon has the effect of rewarding poor experimental design because small group sizes in experiments will tend to produce higher NOELs. Moreover, nonthreshold effects may not be detected due to the low number of animals enrolled or the dose levels applied. In addition, the slope of the dose-response curve is often not considered or may not be determined with sufficient accuracy, and chemicals with steep and shallow dose–response curves are treated alike. Advantages and disadvantages of safety factors in the risk assessment process follow:

Advantages
- Simple application
- Ease of understanding
- Flexibility of use
- Use of expert judgment

Disadvantages
- Uncertainties of threshold values and size of safety factor
- No risk comparison possible
- Slope of dose–response curve not adequately considered
- Experimental NOELs are dependent on group size in the animal toxicity testing and end point selected

One of the most promising alternatives to the use of NOAELs or NOELs is the benchmark concept [194]. In the benchmark approach, a dose–response curve is fitted to the complete

experimental data for each effect parameter. On the basis of the fitted curve, the lower confidence limit of the dose at which a predefined critical effect size is observed (i.e., the dose at which adverse effects start to arise or where 5% of the animals are predicted to be affected, effective dose, or ED_{05}) is defined as the benchmark dose. Advantages of this approach over the NOAEL are:

- The benchmark dose is derived by using all experimental data and gives a better reflection of the dose–response curve.
- The benchmark dose is independent of predefined dose levels and spacing of dose levels.
- The benchmark approach makes more reasonable use of sample size, and better study designs result in higher benchmark doses.

A disadvantage of this method is the uncertainty with respect to the reliability of the approach when results are obtained from toxicity studies performed according to the requirements defined in current guidelines. For the derivation of reliable dose–response relationships, the classical study design of three dose groups and a vehicle control group is limited, since adverse effects may only be observed at the highest dose level. An improved benchmark model fit could be achieved by increasing the number of dose groups without changing the total number of animals in the test.

At present, the determination of a NOAEL is mandatory for risk assessment in the EU. Nevertheless, the benchmark dose method can be used in parallel when a NOAEL cannot be established for the selected toxicological end point because only a LOAEL is available. In this case, benchmark modeling preferred over LOAEL–NOAEL extrapolation, which uses more or less arbitrary assessment factors. Benchmark dose software (BMDS) is available from the US EPA internet site (www.epa.gov).

5.2.4.2. Risk Estimation Techniques for Nonthreshold Effects

The procedures outlined below are most often applied to the low-dose risk estimation of human or animal carcinogens. Since dose–response data are not available for effects in animals at doses relevant to human exposure, extrapolations are required for determining the potential human cancer risk. The methodology employs mathematical modeling to characterize the relationship between exposure and response or to place an upper bound on the dose–response relationship. Dose–response data, available from specific study situations (mainly animal bioassays using high doses of the chemical and, sometimes, heavily exposed population groups), are extrapolated to the often much lower exposures of the general population in order to calculate the possible risk. Therefore, cancer risk assessment generally involves extrapolating risk from the relatively high exposure levels employed in animal studies, or occupational studies where cancer responses can be measured, to risks at the relatively low exposure levels that are of environmental concern. However, since the majority of carcinogenicity experiments use only two or three doses, it is impossible to assess the shape of the dose-response with a reasonable degree of precision. Risk assessment must therefore rely on some arbitrary assumptions about the shape of the dose-response relationship at low doses. Risk estimates thus obtained are not true or actual risk but values obtained by extrapolation well below the range of experimental observations. A summary of the advantages and disadvantages of quantitative risk assessment follows:

Advantages

- Gives numerical values on risk that may be used for setting exposure limits.
- Permits the comparison of risks due to different chemicals.
- Provides a reasonable basis for setting exposure limits by identifying compounds with high risk.

Disadvantages

- Extrapolation of data obtained at high doses to low doses relevant for human exposure by means of mathematical models which are not based on cancer biology and pathophysiology.
- Mechanistic and kinetic data are not used for the risk estimation process.
- Expensive and time- consuming lifelong bioassays are required.

The risk extrapolation techniques used are based on several conservative default assumptions, some unsupported by any direct empirical evidence. Conservatism is introduced to

ensure maximum protection for those exposed to presumed chemical carcinogens. These assumptions were adopted to achieve some consistency in the application of risk-estimating techniques. The major assumptions are (1) carcinogenic risks are estimated from the data obtained in the most sensitive animal model only using positive responses (data from bioassays that do not show a treatment-related increase in cancer incidence are ignored); (2) linear, nonthreshold dose-risk models are applied; (3) statistical upper confidence limits are used rather than best estimates; and (4) a linear dose–response curve is assumed at low doses.

5.2.4.3. Mathematical Models Used in High- to Low-Dose Risk Extrapolation

Mathematical models for quantifying human cancer risk from exposure to carcinogens were first developed in the 1950s. These models were based on the one-hit or the multistage model of chemical carcinogenesis. A probit model was proposed in 1961 for assessing low-dose risk by extrapolation. An improved probit model was introduced in the 1970s by the FDA for computing the level of carcinogenic chemicals permissible in food. This procedure included the suggestion that the dose causing a very low risk (one additional cancer in 1 000 000 exposed people) to be considered as a "virtually safe dose." Introduction of this mathematical model into the risk assessment process stimulated the development of a variety of other mathematical models for carcinogen risk assessment. The basis of all the above methods is to apply a mathematical model to the tumor incidence observed in a long-term animal bioassay.

A major problem in dose– response and risk extrapolation is the determination of an appropriate mathematical model to predict effects at hypothetical low levels of exposure. Several models have been developed, and most of them contain analytical functions that appear to fit the experimental dose range quite well and also contain a dose–response functionality. Most models differ in the functions used to estimate response in the very low dose range (i.e., as dose approaches zero). The mathematical models in common use in carcinogen risk assessment are

- The linear model.
- Statistical or distribution models: log–probit, Mantel–Bryan, logit, Weibull.
- Mechanistic models: one-hit (linear), multihit, multistage (Armitage–Doll), linearized multistage, Moolgavkar model.
- Other models: statisticopharmacokinetic, time-to-tumor.

Linear Model. Linear extrapolation involves the intersection of a straight line between the origin (zero dose) and the upper confidence limit of the response at the single, lowest experimental dose. This model is based on the assumption that the increase in tumor incidence by the applied xenobiotic augments an already proceeding process.

Distribution models are based on mathematical functions of presumed population characteristics i.e. on the assumption that every member of a population has a critical dosage (threshold) below which the individual will not respond to the exposure in question. The probit model assumes that log dose–responses have a normal distribution. This model serves as the basis for the Mantel-Bryan risk extrapolation procedure. Other distribution models on which carcinogenicity dose–response models have been based include the logit and Weibull models.

Mechanistic models are based on the currently presumed mechanisms of chemical carcinogenesis. Each model reflects the assumption that a tumor originates from a single cell. The concept underlying the one-hit model is that a tumor can be induced by exposure of DNA to a single molecule of a carcinogen. This model is essentially equivalent to assuming that the dose–response is linear in the low-dose region and as a consequence tends to produce very low calculated "virtually safe doses" compared with the other currently applied models. The multihit model is a generalized version of the one-hit model which assumes that more than one hit is required at the cellular level to initiate carcinogenesis.

The biological justification for the multistage (Armitage–Doll) model is that cancer is assumed to be a multistage process that can be approximated by a series of multiplicative linear functions. It assumes that the effect of a chemical car-

cinogen occurs in multiple steps and that the effect of each step is additive. The dose–response predicted by this model is approximately linear at low doses and it results in estimates of potential risk that are similar to those of the one-hit model.

The Moolgavkar–Venson–Knudson model attempts a more comprehensive consideration of the biologic processes of cancer formation than the other mechanistic models and may provide a more accurate estimate of human risk by reducing some of the interspecies uncertainty. It is based on a two-stage growth model and considers the birth and death of cells, the effect of cell proliferation on the number of available cells for malignant transformation, and assumes two specific, irreversible, and rate-limiting mutational events to be necessary for cancer development. This model may quantitatively consider major phenomena influencing cancer formation such as cell proliferation, initiation/promotion, genetic predisposition, and synergism/antagonism. Moreover, model parameters may be obtained experimentally. At present, the major limitation for the application of this model is the lack of availability of many of the important biological parameters.

Other Models. The carcinogenicity of many chemicals is based on their biotransformation to reactive metabolites. The statisticopharmacokinetic model arises from consideration of competing metabolic activation and deactivation processes (e.g., detoxification and DNA repair) and estimates the "effective dose", i.e., the level of reactive metabolites formed and interacting with critical macromeolecules, rather than the administered dose.

A modification of the Probit model relates it to the time at which a tumor is detected. For risk assessement, this time-to-tumor model uses the time to observance (latency) in addition to the proportion of animals bearing tumors at each dose.

5.2.4.4. Interpretation of Data from Chronic Animal Bioassays

The results of experimental animal studies may provide a variety of data in addition to the simple indication of the presence or absence of cancer. This information may be useful for the evaluation of potential human cancer risk and includes the number of neoplasms per animal, the number of different types of neoplasms observed, and the number of species affected. The organ or target tissue in which the carcinogenic response occurs is also important, as some rodents have extremely high and variable spontaneous incidence of certain tumor types. Where a chemical increases the number or accelerates the formation of neoplasms which spontaneously occur in high and variable incidence, the response carries less weight than the appearance of tumors with very low spontaneous rates. The time to development of tumor will also give an indication of potency. Further considerations on the interpretation of the obtained data are given in Section 4.8.

5.2.4.5. Problems and Uncertainties in Risk Assessment

The practice of using tumor incidences obtained in long-term animal experiments with high doses (MTD, maximum tolerated dose, see Section 4.8) for human cancer risk assessment and several of the conservative default assumptions used in the extrapolation processes have become the subject of major criticism. Some of the assumptions made when using experimental animal data for human risk assessment are not directly testable experimentally. Nevertheless, these assumptions are widely used because risk assessment would be difficult or impossible without them [188].

However, recent results on the mechanisms of carcinogenesis have revealed a complex biological process with many variables; a comprehensive consideration of all these parameters by a single, generally applicable mathematical model is not possible. Moreover, observations such as nonlinear toxicokinetics and delineated mechanisms of action for nongenotoxic carcinogens have indicated that a more scientifically based approach to carcinogen risk assessment, likely in a case-by- case examination by expert panels, may be more appropriate for defining actual risk than mathematical modeling. The major points of controversy in risk extrapolation from animal experiments and possible solutions to reduce uncertainties are described in Table 41.

Table 41. Uncertainties in quantitative risk assessment and the application of scientifically based methods for reduction of these uncertainties

Uncertainty	Reduction by
Extrapolation between species	physiologically based toxicokinetic modeling, comparative studies on toxicokinetics
Extrapolation from high to low dose	toxicokinetic modeling, quantitative determination of dose – response for biochemical effects of xenobiotics (e.g., DNA adducts, genetic changes), mechanisms of carcinogenesis
Extrapolation from controlled experimental conditions to variable human situations	none
Conservatism	elucidation of mechanism of actions
Toxicity and increased cell proliferation	dose – response for cell proliferation and cytotoxic effects
Dosimetry	toxicokinetic modeling and physiologically based toxicokinetic modeling
Mixtures	research on mechanisms of interactions
Bioassay-inherent factors in risk assessment	expert judgment

Extrapolation Between Species. In the absence of information on the possible mechanism of carcinogenesis by a particular xenobiotic, the tumor incidences observed in an animal experiment are often assumed to be useful in assessing human risk. However, the differences in the physiology and anatomy of laboratory animals and humans are well recognized. For example, the life span of the laboratory rodent is approximately two years whereas that of humans is approximately seventy years. Cancer appears to develop in rodents over a time scale that is proportional to the life span, and it is generally assumed that this will apply to all chemicals being assessed. The assumption that humans will respond in a similar fashion to laboratory animals is frequently shown to be inappropriate. Mechanistic studies demonstrate that qualitative and quantitative aspects of toxicokinetics and biotransformation, DNA repair and tissue susceptibility, and immune and other defense mechanisms may explain observed differences in the response of laboratory species and humans to exposure to carcinogens [195]. Expert judgement may be required to assess the nature of the end point or the mechanisms of carcinogenic action of the chemical in the experimental animal and to decide whether they are relevant to man.

Extrapolation from High to Low Dose. The use of very high doses in animal cancer bioassays and the required extrapolation form high to low dose are a major point of critisim. Most cancer bioassays are performed in relatively small groups of rodents (between 50 and 100) and with administration of high doses. The highest dose selected is usually the "maximum tolerated dose" (MTD) in order to have maximum sensitivity and to ensure that the results obtained do not overlook a carcinogenic response simply because the dose was too low.

The following information that is obtained in bioassays with high doses in animals:

- Definite identification of compounds with carcinogenic activity in the species.
- Information about relative potency of different chemicals.
- Information about the carcinogenic activity of the test chemical, when administered alone.
- Characterization of tumor types, target organs, and presence or absence of dose–response relationship, which permit comparison of different chemicals and help in establishing structure–activity relationships to improve predictive capabilities.
- Information on the lack of carcinogenicity of many chemicals to assist priority setting in public health.

However, they also have the following limitations:

- No direct information about effects at doses lower than dose studied.
- No information on the mechanism of carcinogenicity.
- No information on the effects of the test compound, when administered together with other chemicals (synergistic/antagonistic effects).
- Use of high doses that may cause unspecific toxic effects contributing to carcinogenicity.
- Acute toxic effects that may prohibit long-term administration of specific chemicals in sufficiently high doses to cause tumors.

Human exposure to carcinogens usually occurs at doses several orders of magnitude lower than those used in the experiment. Clearly, the possible shape of the dose–response relationship

is vitally important in establishing the likely effects at doses substantially below those in the observable range. Mathematical models used for extrapolation only give the upper limits of risk, the real risk may be somewhere between zero and the number calculated. Since the dose–response relationship can not be determined experimentally, application of different mathematical models results in markedly different slopes of the curves in the low-dose range (Fig. 69), and this has major implications for risk assessment and the establishment of ADI values.

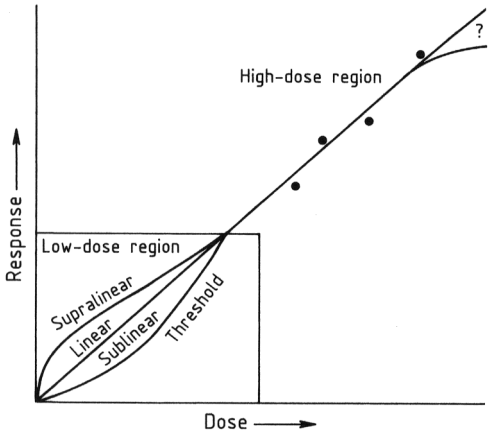


Figure 69. Possible slopes of dose – response curves in the very low dose range below the ability of experimental determination in cancer bioassays
● = Experimental data point

Only one study to determine the actual shape of the dose–response curve at low doses with group sizes large enough to constitute statistical significance was performed, the so-called ED_{01} study using the potent carcinogen 2-acetylaminofluoren as a model compound administered in the diet to mice. Urinary bladder and liver neoplasms were found to be related to 2-acetylaminofluorene administration. The resulting tumor incidences showed that even within one species and with one specific carcinogen, there are major differences in the shape of the dose–response curve at low doses taht depend on the target organ affected. The incidence of bladder tumors at all time points suggested the presence of a threshold for this end point, whereas the dose–response data for the liver tumors was nearly linear at all time points and did not indicate a threshold (Fig. 70).

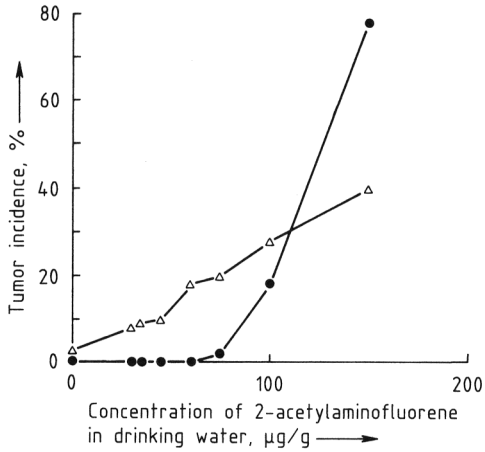


Figure 70. Dose – response data for the induction of liver (Δ) and urinary bladder (●) tumors induced by 2-acetylaminofluorene in mice after 24-month administration in diet

Extrapolation from Controlled Experimental Conditions to Variable Human Situations. In most animals studies, all critical factors are carefully controlled to guarantee the reproducibility of experimental observations. This is in contrast to the sometimes very large differences and time-dependent changes in the environmental exposure and other circumstances of the human population, which cannot be simulated in animal experiments. The complexity of these circumstances (i.e., changing life-style factors or workplaces) would necessitate an assessment of each human individually. Thus, risk assessment normally extrapolates directly from the animal data unless good evidence exists to suggest that an important confounding factor has been introduced. Besides the variable exposure situations, the human populations exposed to chemicals often differ in age, sex, and ethnic background and will certainly have a more heterogeneous genetic makeup than experimental animals. For the majority of carcinogenic risk assessments, individual variability can be taken into account only by the application of safety factors to compensate for these uncertainties.

Dosimetry. The problems of estimating environmental and/or occupational exposure to potential carcinogens have been described above. There are equally difficult problems in extrapolating the doses applied in animal experiments to those which might be encountered by humans

due to environmental or occupational exposure and to distinguish between administered dose and effective dose.

Doses can be extrapolated from animal experiments to humans by five methods:

1) Expression of dose as a function of body weight (mg/kg or mmol/kg).
2) Expression of dose as a concentration in food or water (usually parts per million, i.e., µg/g or µL/L).
3) Expression of dose as a concentration in inhaled air (usually parts per million, i.e., µL/L).
4) Correction of dose for surface area. This is achieved in a complicated process by first raising the body weight of the laboratory animal to the power of 2/3 or 3/4. The correction converts doses that are expressed as concentrations in air or food to milligrams per kilogram of body weight in the laboratory species. The dose is then corrected for body surface area and converted back to concentrations in air or food by using appropriate conversion factors for humans. Corrections using surface area are based on the observation that metabolic rate is proportional to body surface area. For acute toxicity, this method may have some merit, but the rate of biotransformation may differently affect the potency of a carcinogen, depending on the role of biotransformation in detoxification or activation. Using correction factors for surface area provides data suggesting that humans may be more susceptible to potential carcinogens than laboratory animals compared to the data obtained by extrapolation on the basis of body weight.
5) Extrapolation assuming that the tissue dose is the primary determinant of carcinogenic response. This approach requires a study of the pathways of biotransformation and the kinetics of the pathway that generates the ultimate electrophile. In vitro studies may be necessary to obtain appropriate human data.

Further problems occur if the experiment is carried out by a protocol that is completely dissimilar from the human experience, for example, if animal exposure is carried out for a lifetime and human exposure is for a shorter period or if the animal experiment uses an exposure route that is not relevant for humans. As a general rule for chemical carcinogens, where the target tissue dose is presumably the most important determinant of the carcinogenic potency, the total body burden must be computed from the various routes of exposure to assess overall dose.

Mixtures. Cancer bioassays in animals are most often performed with a single chemical of definite high purity; exposure of experimental animals to other chemicals or other confounding factors such as vector-based disease is carefully avoided. In contrast, humans are continuously exposed to mixtures of chemicals and other agents. Thus, even when animals or humans are exposed to two or more chemicals, further complications of data interpretation are introduced. In general, the response of the animal and the human will depend on whether the chemicals' activities are interactive or not and whether the effects of the chemicals are similar or not. If the chemicals are not interactive and the response is similar, an additive effect may be assumed to occur. If they are not interactive, the overall response may be less than additive. In addition, a variety of metabolic or infectious diseases in the course of a human's life may have major impact on the development of a certain tumor due to exposure to the chemical under question. It is not feasible to simulate these real-life factors in the experimental situation [196].

Bioassay-Inherent Factors in Risk Assessment. A variety of biological factors exist that influence the assessment of carcinogenic hazard. Among these are the quality of the experiment, including the quality of the pathology, environmental control of animal facilities, estimation and standardization of chemical administration, purity of the chemical administered, and many other factors that together make up compliance with good laboratory practice. A further problem may be encountered if differences occur in outcome from different experiments. In the majority of cases, some aspect of an exceptional experiment may explain why the response was different (e.g., use of a different strain of animal). Since only positive data are used and negative data are ignored for carcinogenic risk assessment purpose to provide a conservative estimate of risk, this factor may also contribute to an overestimation of risk.

Conservatism and the Mechanisms of Chemical Carcinogenesis. To provide maximum protection, a conservative approach to risk assessment is preferred. This approach assumes that a single molecule of a carcinogen can interact with DNA and produce cancer; therefore, there can be no threshold for chemical carcinogenesis. Moreover, selection of the most sensitive response, irrespective of mechanism of action, and the assumption that the dose–response is linear at low doses may overestimate risk. This overestimation may be due to the unknown degree of conservatism at each step and to the amplification of previous bias in the assumption by the next step. The magnitude of overstatement of risk by these conservative assumptions is unknown, but is claimed to amount to several orders of magnitude.

Role of Toxicity and Increased Cell Proliferation. Critics point out that the high doses used in rodent carcinogenicity studies may have nonspecific effects such as an increase in the rate of cell proliferation due to cytotoxic effects [197]. These effects can be unique to high doses, and mitogenesis may itself be mutagenic in numerous ways, either by errors in replication or by conversion of endogenous DNA damage (e.g., by oxidative processes) and exogenous DNA damage to mutations before repair can occur. Moreover, sustained increases in the rate of cell proliferation may also yield secondary mutational events and could be important in the promotional phase of carcinogenicity by increasing the clonal expansion of initiated cells and thus increasing the chance that multiple critical mutational events will occur [198]. Thus, cell division will increase the chance of tumor formation. In this case, the tumor incidence for the same chemical applied at much lower doses is likely to be much lower than a linear model would predict and may even be zero [165, 199, 200]. Therefore, it would be important to add methods for determining cell division to animal cancer bioassays and apply the obtained results to estimate low-dose risks more adequately. Cell proliferation has been implicated as a major contributor to the cancerogenicity of several chemicals such as phenobarbital, 1,4-dichlorobenzene, *d*-limonene, and peroxisome proliferators.

Selection of Mathematical Model. The various models used in risk extrapolation fit the experimentally observed data well; however, they predict widely differing potential risks at low doses. Depending on the data used in the calculation, the predicted risks may differ by several orders of magnitude. These differences are inherent in the application of mathematical models, since only two or three doses are used in the experimental dose groups.

5.3. Future Contributions of Scientifically Based Procedures to Risk Assessment and Qualitative Risk Assessment for Carcinogens

Since quantitative cancer risk assessment can neither claim to be a scientific basis for development of historical models nor attempt to incorporate the large amount of scientific data on the mechanism of carcinogenesis into the assessment of risk, qualitative approaches considering all relevant data may be the best available solution for risk assessment. This approach, referred to a "weight-of-evidence determination of risk" is increasingly emphasized by regulatory authorities. The weight-of-evidence approach includes critical evaluation of the animal bioassay and all other available information on adverse effects of the chemical together with biotransformation, toxicokinetics, and expert judgment [201].

The criticims outlined above have demonstrated the need for further refinement of the extrapolation procedures by toxicokinetics and studies on the mechanisms of tumor formation. The role of animal experiments in predicting the potential risk of human carcinogen exposure will

Table 42. Descriptive dimensions proposed as a framework to facilitate the use of mechanistic data in evaluation of carcinogenic risk to humans

Data set	Example of information required
Evidence of genotoxicity	DNA adduct formation, mutagenicity, bioactivation
Evidence of effects on the expression of genes relevant to the process of carcinogenesis	alterations of the structure or quantity of product of a proto-oncogene or suppressor gene
Evidence for effects on cell behavior	mitogenesis, cell proliferation, hyperplasia
Evidence of time and dose – response relationships and interactions	initiation, promotion, progression

likely remain an important step of the risk assessement process despite the obvious limitations of this approach [197, 202]. However, the data obtained with animals must be interpreted with caution and in light of other data, both quantitative and qualitative, on the adverse effects of the compound (Table 42).

Application of Toxicokinetic Models in Risk Assessment. Information on the way in which a chemical is absorbed, biotransformed, and excreted may be critical in extrapolating the relevance of the results obtained in experimental animals to humans [130, 203]. For the purposes of risk extrapolation, it is generally assumed that the administered dose is proportional to the effective dose. However, many chemicals are known to be carcinogenic only after they have been activated to reactive electrophiles by enzymatic reactions. The amount of reactive metabolite formed might not be directly related to dose because saturable enzymatic processes are involved in both bioactivation and detoxication. The extent of bioactivation and detoxication may therefore be highly dose dependent, and the relationship between administered dose and target dose may not be linear at all administered doses. For example, when high-affinity, low-capacity enzymes catalyze the detoxication of a xenobiotic and low-affinity, high-capacity enzyme catalyze its bioactivation, the amount of reactive metabolite formed is likely highly dependent on the administered dose (i.e., a tenfold higher administered dose may result in a 100-fold higher effective target dose). Moreover, there may be depletion of cosubstrates required by the enzymes catalyzing bioactivation or detoxication. After the reactive metabolite is formed, it is often deactivated by a second enzyme, such as epoxide hydrolase or glutathione *S*-transferase. These enzymes can also be saturated. The reactive metabolites that are not destroyed by these detoxication pathways may bind to DNA. The metabolites bound to DNA can be removed by saturable DNA repair systems. These effects are referred to as nonlinear toxicokinetics. Nonlinear kinetics are also seen with chemicals inhibiting or inducing drug-metabolizing enzymes. Application of a carcinogen at high doses may result in the induction of enzymes catalyzing its bioactivation and may leave detoxifying enzymes unaffected. Thus, the expected steady-state concentrations of reactive intermediates present in the cell and capable of binding to DNA are expected to be disproportionately higher after application of high doses which induce biotransformation enzymes than after low doses which leave the levels of biotransformation enzymes unaffected. Moreover, the metabolism of a xenobiotic may be changed as a consequence of the effects of long-term administration of the chemical [204].

To estimate the dose-dependent relationship between administered dose and the effective dose, toxicokinetic models incorporating saturable processes have been applied. The examples in Figure 71 show the theoretically derived relationships between administered dose and effective dose for the same chemical in animals by varying the kinetic parameters for bioactivation, detoxication, and DNA repair [205]: saturation of enzymatic bioactivation (A), saturation of detoxication and activation (B), and saturation of activation, detoxication, and DNA repair (C).

The hockey-stick shape of the dose-response curve may remain unnoticed in animal experiments with high dose. The nonlinear correlation between effective dose and administered dose in the low, relevant dose region for human exposure may result in a decrease in the potential risk of exposure when compared to risk estimation based on experiments with high doses. Effective doses may be determined by measuring the amount of DNA adducts formed after administration of a carcinogen based on the assumption that for genotoxic agents the carcinogenic response is related to the extent of DNA adduct formation in target tissues. Unfortunately, nonlinear dose–response curves for effective doses have not yet been observed experimentally; all attempts to determine the dose-dependent formation of DNA adducts of potent carcinogens have shown linear relationships between administered dose and effect [206]. However, the increased sensitivity of analytical instruments currently available offers the opportunity to study the dose–effect curve for a wide range of carcinogens transformed to intermediates with different electrophilic reactivity in the low-dose range by quantifying the dose-dependent concentrations of DNA lesions. Quantitation of xenobiotic–hemoglobin adducts and chromosomal abnormalities in lymphocytes (e.g., sistechromatid exchange frequency) offers other examples of biological markers that may prove

useful in the definition of dose–response curves. The application of these procedures offers the advantage of obtaining quantitative information on a nonstochastic effect thought to be relevant in the mechanisms of carcinogenesis and thus in risk assessment.

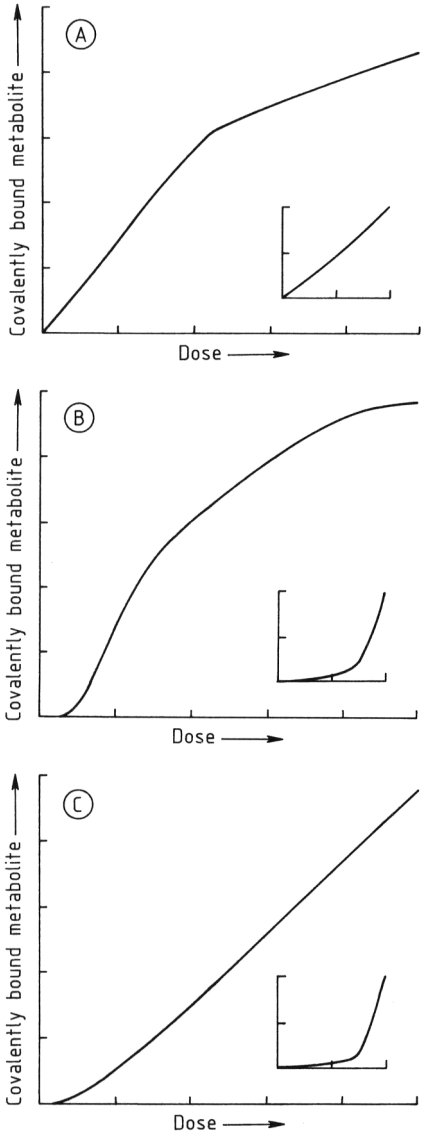


Figure 71. Possible relationships between administered dose and effective dose for the same data set including correction for nonlinear toxicokinetics
A) Saturation of enzymatic bioactivation; B) Saturation of detoxification and activation; C) Saturation of activation, detoxification, and DNA repair
The shapes of the curves in the low-dose range are shown in detail in the small sections.

As noted in Section 5.2.4.5, dosimetry may be a major cause of uncertainties in risk extrapolation form animals to humans. Besides saturable enzymatic reactions, the effective dose of a carcinogenic chemical may also be influenced by species differences in absorption, distribution and elimination, which may also be influenced by the dose administered.

To account for such factors, physiologically based pharmacokinetic models are increasingly used in the process of risk assessment. The principal purpose of the application of physiologically based pharmacokinetic models is to predict the concentration of carcinogen at the target site and describe the relationship between administered dose and target dose over a range of concentrations. By application of these models, a more accurate dose extrapolation is possible over broad ranges and may also incorporate nonlinearities in bioactivation and detoxification. Since the principles employed in the development of these models apply across species, the definition of the relationship between administered dose and effective dose and the important rate processes that cause a deviation from linearity permits a more accurate species extrapolation. The application of known physiological parameters may also enable the prediction of target organ concentrations in humans when direct measurements are not possible and may permit the comparison of different routes of application for effective dose. Physiologically based pharmacokinetic models are increasingly used to support quantitative risk assessments. Due to the risk estimates based on effective dose rather than external dose levels and the consideration of potential nonlinear relationships, an overestimation of risk obtained by linear extrapolations may be overcome and result in more scientifically founded risk assessments.

Mechanisms of Carcinogenicity and the Risk Assessment Process. Research on the molecular effects of particular agents has increased our understanding of the mechanisms of carcinogenicity. A range of biological processes has been implicated in carcinogenesis. Some of these mechanisms may be common to most carcinogens, others may be restricted to particular classes of chemicals or specific circumstances. In spite of the incomplete knowledge on carcinogenesis and a limited understanding of many

processes, mechanism.based decisions are increasingly being introduced into risk assessment [207].

The emerging field of molecular toxicology may hold promise for the future of human risk assessment, and the application of methods developed for molecular biology may also offer more accurate ways of assessing human risk. The most promising application of mechanistic results for risk assessment are non-genotoxic carcinogens [208]. A number of xenobiotics are carcinogenic in animals but do not cause any detectable mutagenicity in in vitro studies. In contrast to other chemical carcinogens, which affect multiple organs or both sexes of both rat and mouse, many nongenotoxic carcinogens affect only a single organ in a single sex of a single species [133]. There are numerous examples of xenobiotics or treatment regimens in which production of tumors in animal experiments unlikely involves interaction of the xenobiotic or its metabolite with DNA. Examples include the induction of subcutaneous sarcomas by the repeated injection of glucose or saline solutions, the induction of skin cancer after chronic skin damage, and the induction of bladder cancer by the implantation of solid materials [209]. Recent experimental evidence also suggests that a number of xenobiotics that do not damage DNA may induce cancer in specific organs after systemic administration. Examples include chemicals binding to the circulating protein α_{2u}-globulin [210], peroxisome proliferators, and several structurally unrelated compounds interacting with receptors [211] (Table 43).

Table 43. Examples of nongenotoxic carcinogens and their presumed mechanisms of action

Class of nongenotoxic carcinogen	Typical examples	Presumed mechanism of action
Peroxisome proliferators	diethyl-hexylphthalate	receptor-mediated increase in gene transcription, oxidative stress
α_{2u}-Globulin-binding agents	unleaded gasoline, limonene, decalin	regenerative cell proliferation due to cytotoxicity
Phenobarbital		mitogenic activity
Chlorinated dioxins	2,3,7,8-tetrachloro-dibenzodioxin	receptor-mediated increase in gene transcription
Hormones	17-α-ethynyl-estradiol	receptor-mediated increase in gene transcription

The terms "genotoxic" and "nongenotoxic" were defined by Butterworth [133]: "A genotoxic agent is one for which a primary biological activity of the chemical or a metabolite is alteration of the information encoded in the DNA. These can be point mutations, insertions, deletions or changes in chromosome structure or number. Chemicals exhibiting such activity can usually be identified by assays that measure reactivity with the DNA, induction of mutations, induction of DNA repair or cytogenetic effects. Nongenotoxic chemicals are those that lack genotoxicity as a primary biological activity. While these agents may yield genotoxic events as a secondary result of other induced toxicity, such as forced cellular growth, their primary action does not involve reactivity with the DNA."

Xenobiotics such as unleaded gasoline, limonene, and 1,4-dichlorobenzene induce renal cancer in male rats, but not in female rats or in either sex of mice [212]. Metabolites of these xenobiotics bind to the circulating protein α_{2u}-globulin, whose synthesis in the rat is under the control of androgens. The modified α_{2u}-globulin is concentrated in the kidney proximal tubules and is not degradable by the processes responsible for degradation of unmodified α_{2u}-globulin. Thus, accumulation of the modified protein causes cytotoxicity and regenerative cell proliferation, which has been implicated as the cause of tumor formation in the kidney.

Tumors in organs whose function is regulated by the endocrine system are often observed after hormonal therapies in humans and hormone treatment in animals and, due to the absence of genotoxicity of most hormones, nongenotoxic processes involving receptor-mediated transcription and activation or repression of specific genes have been implicated in hormonal carcinogenesis [213].

For both of the above cited examples, threshold mechanisms may be postulated. Kidney cancer induced by α_{2u}-globulin-binding agents requires cytotoxicity and cell death. Nontoxic low concentrations of these xenobiotics applied may not be tumorigenic. For receptor-mediated processes, a disproportional relationship between receptor occupancy and hormonally mediated cancer is also considered likely [214–216]. Hence, any assessment of carcinogenic

risk of these chemicals must take into account the mechanisms by which they produce their effects.

The use of molecular toxicology in risk assessment is also advocated by the International Agency for Research on Cancer [207]. A monograph on the identification of cancer risks by mechanistic investigations suggested that cancer risks by mechanistic investigations suggests that "when available data on mechanisms are thought to be relevant to evaluation of the carcinogenic risk of an agent to humans, they should be used in making the overall evaluation, together with the combined evidence for animal and/or human carcinogenicity." The consensus report further states that no definite guidelines for the inclusion of mechanistic data in the evaluation of carcinogens can be elaborated. However, a range of options are available: "First, information concerning mechanisms of action may confirm a particular level of carcinogen classification as indicated on the basis of epidemiological and/or animal carcinogenicity data. Second, for a particular agent, strong evidence for a mechanism of action that is relevant to carcinogenicity in humans could justify 'upgrading' its overall evaluation. Third, an overall evaluation of human cancer hazard on the basis of animal carcinogenicity data could be downgraded by strong evidence that the mechanism responsible for tumor growth in experimental animals is not relevant to humans. In keeping with the goal of public health, priority must be given to the demonstration that the mechanism is irrelevant to humans."

5.4. Risk Assessment for Teratogens

The timing of exposure and patterns of dose–response from animal studies have important implications for extrapolating animal data to humans. A wide spectrum of end points can be produced, even under the controlled conditions of timing and exposure that can be achieved in animal studies. In some cases, the spectrum includes a continuum of responses, with depressed birth weight or functional impairment occurring at low doses, birth defects at intermediate doses, and lethality at high doses. Less commonIy, birth defects alone or lethality alone are produced. Therefore, in estimating human risk, all exposure-specific adverse outcomes must be taken into consideration, and not just birth defects.

A similar response pattern is observed in humans exposed to developmental toxicants. The spectrum of responses is determined by the time and duration of exposure, magnitude of exposure, and interindividual differences in sensitivity. Hence, manifestations of developmental toxicity can not be expected to be identical across species; that is, an animal model can not be expected to forecast exactly the human response to a given exposure. For instance, an agent that induces cleft palate in the mouse may elevate the frequency of spontaneous abortion or retard growth in humans. However, any manifestation of exposure-related developmental toxicity in animal studies can be regarded as indicative of a spectrum of response in humans.

Epidemiological data suggest that the majority of human embryos with chromosomal and/or morphologic abnormalities are lost through early miscarriage and that relatively few survive to term. Consequently, determination of malformations or growth retardation at the time of birth alone (malformations, stillbirths, low birth weight) is likely to result in a substantial underestimate of the true risk, since the onset of embryolethality would be missed.

The sensitivity (ability to detect a true positive response in humans) and specificity (ability to detect a true negative response in humans) of laboratory animal studies have been evaluated [217]. Of 38 compounds with demonstrated or suspected teratogenic activity in humans, all but one (tobramycin, which causes otological deficits in humans) tested positive in at least one animal species. Approximately 80% of the compounds were positive in multiple species. A positive response was elicited to 85% in the mouse, 80% in the rat, 60% in the rabbit, 45% in the hamster, and 30% in the monkey. These findings indicate that the usual laboratory animal species are highly sensitive for detecting human teratogens.

In contrast to the high sensitivity, laboratory animals show low specificity for predicting human teratogenesis; of 165 test compounds with no evidence of teratogenic activity in humans 65 (41%) were positive in more than one animal species. The high percentage of false positive results (i.e., compounds with no evidence for human teratogenicity inducing malformations in

animals may be in part accounted for by the high doses usually applied in the animal tests. Also, human studies determine effects from the time of birth onward, which may result in underestimation of the true risk.

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