Department of Health

Report on Health and Social Subjects

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Guidelines for the Evaluation of Chemicals for Carcinogenicity

Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment

London: HMSO

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Preface

In the last decade there has been continuing interest in the possibilities of preventing cancer. It is clear that some cases of cancer in man are causally associated with exogenous chemicals. Once identified, exposure to these agents can often be controlled and a reduction in the incidence of the associated cancer may be expected. The previous Guidelines for the Testing of Chemicals for Carcinogenicity published in 1982 discussed in detail animal studies necessary for the detection of carcinogenic substances.

Animal testing procedures are now well established. The Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment have taken the opportunity of preparing new guidelines which address the wider issue of evaluating chemicals for carcinogenic potential in humans. This is based on many sources of information, including epidemiology, structural chemistry, metabolic studies and short term mutagenicity tests, as well as the results of long term animal testing.

I am grateful to the Chairman and members of the Committee for their wide-ranging review as embodied in this document. By reflecting current thinking and practices these revised guidelines will be of value to those who make decisions in this important area of preventive medicine.

E D Acheson
Chief Medical Officer
Department of Health
July 1991

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Contents

Summary

Chapter 1 gives some background information on general issues in chemical carcinogenesis. It briefly discusses mechanisms by which genotoxic and non-genotoxic substances may be involved in the development of cancer. The role of oncogenes and tumour-suppressor genes in molecular carcinogenesis is also described.

The contribution from epidemiological studies to the overall assessment is dealt with in Chapter 2. The relative merits and limitations of different types of epidemiological investigations are discussed.

Chapter 3 considers the major classes of chemical carcinogens with regard to the different mechanisms by which they exert their carcinogenic effects. The rôle of metabolism is considered.

Chapter 4 covers the use of short-term predictive tests for screening for carcinogenic potential of chemicals (mutagenicity tests and cell transformation assays). Reference is made to the strategy for mutagenicity testing given in the Committee on Mutagenicity's 'Guidelines for the Testing of Chemicals for Mutagenicity', which is also relevant to predictive short-term testing for carcinogenicity.

The main points to be considered in designing a carcinogenicity bioassay are covered in Chapter 5, and some of the problems which might be encountered during the performance of such a study are discussed. Special problems associated with the carcinogenicity testing of certain classes of substances are reviewed.

The interpretation of the results of carcinogenicity studies is covered in Chapter 6. Statistical methodology is not dealt with in detail, but reference is made to more specialized guidelines. Advice is given on the problems of interpreting the biological significance of results. A number of factors which can influence the interpretation of the results of a study (confounding factors) are examined. Mechanisms of carcinogenicity are discussed in the context of interpreting the relevance to humans of a carcinogenic response in animals.

Assessment of the hazards and risks from exposure to chemical carcinogens is discussed in Chapter 7. It explains the COC's differential handling of carcinogens, depending on whether or not a threshold level of exposure can be set for the chemical's carcinogenicity. It is proposed here and in earlier Chapters that threshold levels of exposure (below which there is no carcinogenic hazard) can reasonably be set for many non-genotoxic carcinogens if their modes of action are understood but not for genotoxic carcinogens (which are assumed to present

a finite carcinogenic risk at any level of exposure). Methods of quantitative risk assessment of exposures to non-threshold (presumed genotoxic) carcinogens are briefly presented, and the COC's reasons for not using such methods are set out. The way in which the acceptability (or otherwise) of human exposure to chemical carcinogens is assessed by UK regulatory authorities is summarised with particular reference to the rôle of the COC.

References and suggestions for further reading are given at the end of individual chapters. A Glossary is provided and the Committee's terms of reference are appended.

Introduction and scope of guidelines

The last set of guidelines drawn up by the Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment (COC) was published in 1982. They dealt in the main with the design, conduct and interpretation of long-term animal bioassays. Since these tests have now become reasonably well standardised, it seemed appropriate to use this new edition to address the overall evaluation of chemicals as potential human carcinogens in a more comprehensive fashion.

Animal bioassays still form an important part of the text, but the guidelines have been broadened to include an introductory chapter on general aspects of chemical carcinogenesis and further chapters on epidemiology, short-term predictive tests and approaches to risk assessment. It is not the Committee's purpose to set out procedures which must be inflexibly followed; other guidelines from appropriate regulatory authorities lay out in detail the required procedures for testing and it is fortunate that much of the data required for such documents are becoming more standardised. The emphasis of these guidelines is deliberately directed to some of the problems that are encountered in appraising potential human carcinogens for regulatory purposes. Some of the issues considered are acknowledged to be controversial and reasonably-argued interim opinions have sometimes had to stand in the place of definitive answers.

Evaluation of a chemical for carcinogenicity will usually be required:

- —where a substance to which man is, or may be, exposed has a chemical structure that suggests carcinogenic potential.
- —where large numbers of people may be exposed, or a small number heavily exposed, especially over a prolonged period of time.
- —where a substance causes concern as a result of some specific aspects of its biological and other effects, observed in previous tests. Examples include prolonged retention in tissues or particular patterns of toxicity.

The necessity for evaluation (for example, whether a specific compound is expected to produce high, or moderate prolonged, exposure) would normally be decided on a case-by-case basis by the appropriate regulatory authorities having regard to other relevant data.

1. Some general issues in chemical carcinogenesis

Cancer ranks third among the causes of death in England and Wales and in 1988 fatal cancers accounted for 25% of the total deaths recorded by the Office of Population Censuses and Surveys. Given the problems of successful treatment of the established disease, there is increasing emphasis on early detection and prevention.

Many chemicals are known to be capable of causing cancer in experimental animals and some have been found to do so in humans. The study of cancer deaths in the United States, published by Doll & Peto in 1981, is still the most authoritative analysis of the preventable causes of human cancer in an industrialized society. Known factors or classes of factors were grouped into 11 categories and 'best estimates' were calculated for the percentage contribution which each made to the total numbers of cancer deaths. *Geophysical factors* (ionizing radiations, ultra-violet light) accounted for an estimated 3% of these deaths, and *infections* for 10%—a larger proportion would be expected in parts of the world with a high incidence of virus-associated cancers such as hepatocellular and nasopharyngeal carcinomas. Of the remaining 9 categories, *tobacco* emerged as the single most important human chemical carcinogen with an estimated contribution of 30% to all cancer deaths. Estimates for another 5 groups where exogenous chemicals could be implicated were much lower: *occupation* (4%), *pollution* (2%), *industrial products* (2%), *medicines* (< 1%), *alcohol* (3%). Cancer deaths attributable to *diet* were estimated at approximately 35% but the authors stress the difficulties in evaluating this category: in an industrialized society non-genotoxic dietary agents and total caloric intake are likely to be more important than the presence of potent carcinogens such as aflatoxin. The estimated contribution from *food additives* was low: less than 1%. *Reproductive and sexual behaviour*, accounting for an estimated 7% of cancer deaths, is likely to be due mainly to endogenous hormonal dysfunction rather than to exogenous chemicals. The reader is referred to the original text for details of the definitions of the various proposed aetiological factors and classes of factors, and for the basis on which the 'best estimates' were calculated; but enough information has been extracted to provide a broad context in which the relative contribution made by chemicals to the overall causes of human cancer can be set.

The evidence that a given chemical is carcinogenic is drawn from several sources. Epidemiological studies provide direct information for segments of human populations exposed to the suspect carcinogen. The biological effects of a putative carcinogen can also be examined in life-time exposure studies in laboratory animals and, for certain groups of chemicals, in short-term labora-

tory tests for genotoxicity. Additional information may be gained from an examination of the chemical structure of the compound and the metabolic changes which it may undergo in the body. These various categories of evidence form the basis for evaluating hazards and estimating risks of potential chemical carcinogens for humans; and they are discussed, together with their inherent advantages and limitations, in the chapters that follow. The rest of this chapter will consider some of the more fundamental issues and problems that are likely to arise when the carcinogenic potential of any chemical substance is being considered. Discussion is deliberately brief: the subject is changing rapidly and any detailed appraisal may soon be made obsolete by new information.

There are, to begin with, several problems with terminology. *Initiation, promotion,* and *progression* are widely used terms which were originally devised and applied to laboratory models of 2- and 3-stage carcinogenesis, based mainly on skin painting experiments in mice. They continue to be used in different and wider contexts, and the definition of promotion (in particular) has become increasingly problematical—see page 7. The categories of *genotoxic* and *non-genotoxic* carcinogens are very widely used but, again, definitions are not straight-forward. A distinction can, for example, be drawn between compounds which directly damage DNA and those which damage DNA indirectly—for instance, by causing the production of chemicals which generate H_2O_2 or free radicals or interfere with topoisomerases. Some genotoxic compounds also exert effects which are typical of non-genotoxic agents—acting, for example, as stimulants of cell proliferation. The existence of non-genotoxic carcinogens is now beyond reasonable doubt, but it remains difficult to define them except in a negative fashion—that is, as tumour-inducing chemicals which show no activity in a series of well-conducted, fully-validated genotoxicity tests.

Genotoxic agents

The carcinogenic potential of genotoxic compounds can often be inferred from a knowledge of the chemical structure of the parent substance and/or its metabolites, and its capacity to act as a mutagen in a set of short-term assays. Active genotoxic agents are electrophilic and react with DNA bases, principally in the form of covalently-bound adducts. Knowledge of the generation of electrophilic ultimate carcinogens and the adducts that they form with guanine and other DNA bases has accrued over the years, but other modes of interaction between genotoxic carcinogens and DNA have been recognized more recently. Some anthracycline drugs, for example, interact with DNA by intercalation—the physical binding of an agent such that it becomes wedged between the stacked bases of the DNA double helix. Disruption of topoisomerase II is likely to ensue, causing chromosome aberrations which are independent of cell-cycle stage; DNA and RNA polymerases are also inhibited.

A cell which has sustained DNA damage may respond in several ways. It may be restored to normal by the operation of error-free DNA repair. It may fail to divide further or die because, for example, a cross-linking adduct prevents replication of DNA or synthesis of an essential protein. Thirdly, it may survive

with imperfectly-repaired DNA. Such a cell will be capable of further division but will transmit altered DNA, in the form of one or more mutations, to daughter cells. The essential feature of what are known as error-prone repair systems is that the normal rules of complementary base-pairing are somehow suspended in order to allow DNA replication to proceed on a damaged template. As a result, wrong bases are inserted into the newly synthesized DNA strand; the cell survives but incurs a high level of mutation. Recombination of DNA strands during replication of damaged DNA may also contribute to the increased levels of mutagenicity.

Mutations are of 3 general types: point mutations, chromosomal mutations and genomic mutations (see Table 1.1). It is only within the last decade that studies in molecular biology and molecular genetics have begun to identify the particular genes which are altered or rearranged as a consequence of carcinogen-inflicted mutations. Put very simply, the main areas of interest relate to oncogenes and tumour suppressor genes.

Oncogenes are activated forms of proto-oncogenes—a highly conserved, ubiquitous group of genes which encode proteins that regulate normal growth and differentiation in eukaryotic organisms. They do so in various ways, functioning as growth factors, growth factor receptors, signal transducers, protein kinases and transcriptional activators. At least 5 different mechanisms

Table 1.1: *Types of mutation*

Point mutation a change in the nucleic sequence in one or a few codons which occurs either by base-substitution (one base is substituted by another) or by deletion or addition of one or more bases from one or more codons. Additions or deletions change the reading frame of the DNA and are known as frameshift mutations. Point mutations resulting in a codon change which specifies the insertion of the wrong amino acid into a polypeptide are called missense mutations. A missense mutation may lead to the production of a defective protein if it occurs at a critical site in a polypeptide—for example, at the active centre of an enzyme, or at a site at which a polypeptide should normally fold. A point mutation which converts a codon normally specifying an amino acid to one which is a terminator codon causes polypeptide synthesis to stop prematurely, so that defective proteins are produced which contain short polypeptide fragments. Mutations of this type are called nonsense mutations.

Chromosomal mutation a morphological alteration in the gross structure of chromosomes, and is usually detected by microscopic examination of fixed and stained cells at metaphase. Additional, more subtle changes are more easily seen if the chromosomes are 'banded' by special staining methods. Each chromosome in a karyotype has a banding pattern which is characteristic for the individual chromosome of the given species. Chromosomal mutations result from breakage and reunion of chromosomal material during the cell cycle, and include inversions (a length of chromosome is inserted back-to-front) and translocations (one section of chromosome becomes attached to another). The loss or repositioning of DNA that ensues may have profound consequences for gene expression, and in many cases chromosomal mutations are lethal to the affected cell or individual. There is growing evidence that chromosomal mutations can be of critical significance in carcinogenesis.

Genomic mutation a change in the number of chromosomes in the genome. The normal diploid genome is euploid, and contains a complete set of chromosomes from each parent. Polyploidy occurs where the diploid genome is doubled or tripled. Loss or gain of a single chromosome is known as aneuploidy, and may occur as a result of non-disjunction during cell division. Addition of 1 chromosome is trisomy, deletion of 1 chromosome is monosomy. Like chromosomal mutation, aneuploidy is regarded as an important element in carcinogenesis.

Table 1.2: *Mechanisms of activation of proto-oncogenes*

Activation of proto-oncogenes
At least 5 mechanisms now identified
(1) point mutation within the oncogene
(2) chromosomal rearrangement resulting in formation of a new gene product, part of which consists of sequences from the proto-oncogene
Mechanisms (1) and (2) result in *qualitative* changes to proto-oncogenes—in effect, the creation of new genes which over-ride the functions of the normal genes from which they are derived.
(3) chromosomal rearrangements which place a proto-oncogene next to an inappropriate promoter of mRNA transcription
(4) addition of a strong promoter of mRNA transcription from a virus to an oncogene
(5) increase in the number of copies of the proto-oncogene in the cell—gene amplification
Mechanisms (3) to (5) lead to *quantitative* changes—normal proto-oncogenes make abnormally high levels of their gene products.

have now been identified by which normal proto-oncogenes are converted into oncogenes, all of them involving modification to DNA structure or function—see Table 1.2. Activation of proto-oncogenes by these mechanisms is a dominant-like effect such that the cell acquires genes or gene products which over-ride or add to the genes or gene products that are normally expressed.

Oncogenes have been identified in several different tumour types in humans and in laboratory animals, but certain cautionary points should be noted. At present, the occurrence of many oncogenes is variable within and between tumour categories and, in some neoplasms, a given oncogene may occur rarely or not at all. The occurrence of specific oncogenes is not distinctive for different classes of chemical carcinogens. The contribution of increased and/or abnormal gene products to the carcinogenic process is largely unknown.

One important line of work indicates that proto-oncogenes are activated at several different points during the development of tumours, such that a succession of different mutagenic events is likely to have taken place.

Tumour-suppressor genes (anti-oncogenes, recessive oncogenes) differ from oncogenes in that they are involved in carcinogenesis only when they are deleted or inactivated—in other words, when there is an effective *loss* of gene function. The most fully documented example is the Rb gene which is lost or mutated in the childhood tumour retinoblastoma. The gene has now been cloned and, when inserted into retinoblastoma cells *in vitro*, it will restore normal function. Inactivation of the Rb gene has also been demonstrated in several adult cancers such as carcinomas of the breast, lung and prostate. The gene product, a phosphoprotein known as p-105-rb, has been identified and shown to form physical bonds with oncoproteins coded by nuclear oncogenes found in 3 classes of human DNA viruses—observations which point to ways in which oncogenes and tumour suppressor genes may interact.

Other partly-characterised tumour suppressor genes include p53, thought to be a frequent target for mutation in various common human tumours including lung cancers associated with smoking; and the DCC gene whose expression is absent or greatly reduced in colorectal cancer (deleted in colorectal cancer). Somatic mutations, including deletions, point mutations and DNA insertions, have been detected in the DCC gene in this tumour.

The major focus of interest now is to clarify the interaction between oncogenes and tumour suppressor genes. The complexities are illustrated by human colorectal cancer in which at least 4 sequential genetic alterations have been identified in the course of tumour development—activation of *ras* proto-oncogenes (eg K-RAS on chromosome 12) and loss of suppressor genes on chromosomes 5, 17 and 18.

Non-genotoxic agents

There is a gross discrepancy in our current knowledge of non-genotoxic agents compared with the genotoxic agents considered in the preceding section. Their modes of action are diverse and often imperfectly documented at descriptive, let alone at mechanistic, levels. No consistent 'alerts' are provided by their chemical structures. There are no generally accepted short-term screening tests.

Certain broad comments can, however, be made although exceptions can be set against many of them. Non-genotoxic agents typically exert carcinogenic effects at large doses over long periods of time. The effects are initially reversible. They tend to show species and target organ specificity. Some non-genotoxic agents (phorbol esters, TCDD, hormones) act through receptors, while others (saccharin, certain antioxidants, several hepatotoxic and nephrotoxic compounds) do not. Apparent no-effect levels can often be demonstrated which carry important implications for determining threshold effects (see Chapter 7).

The most clearly understood effects of non-genotoxic agents are on cell proliferation where they may act as mitogens, cytotoxins or as more subtle perturbers of the normal process of growth control eg interfering with endocrine feedback control in the thyroid. The net effect is progressive disturbance of physiological homeostasis within the target tissues, culminating in the development of tumours. It is essential that stimulation is appropriately intense and prolonged, and that there are adequate numbers of susceptible target cells that are capable of responding. Repeated cycles of cell damage (or death) and cell regeneration appear to be particularly important, and have been well demonstrated in target organs such as the liver and kidney. Non-genotoxic agents may damage lysosomes and stimulate peroxisomes and endoplasmic reticulum. A few examples of non-genotoxic chemicals which induce tumours in the liver, kidney, thyroid, bladder and stomach are listed in Table 1.3.

There is evidence that, compared with normal cells, chronically proliferating cells are at a greater risk of sustaining DNA damage and are probably less able to repair it by error-free processes; the longer the period of mitotic stimulation,

Table 1.3: *Examples of non-genotoxic agents with carcinogenic effects*

LIVER

Phenobarbital, dieldrin, aldrin, γ-HCH, DDT, DEHP, TCDD, halogenated biphenyls, hypolipidaemic agents, ethinyl oestradiol

Carcinogenic effects rather more common in mice than rats. Substances stimulate proliferation of endoplasmic reticulum (microsomal enzymes) and, in some instances, peroxisomes. Adaptive growth and proliferation of liver cells: hypertrophy and hyperplasia with (usually) little necrosis.

Carbon tetrachloride, chloroform, tri- and tetrachloroethylene

Carcinogenic effects principally seen in mice.
Major effect is cytotoxicity, provoking regenerative/reparative hyperplasia.

KIDNEY

Chloroform

Direct cytotoxic effect on renal tubules, inducing tumours in rats (males > females) but not mice.

Tri- and tetrachloroethylene, pentachloroethene, 1,4-dichlorobenzene, unleaded gasoline

Affect male rats only through *α2μ-globulin nephropathy*. α2μ-globulin: low m.w. protein produced in liver of male rats only. Filtered at glomeruli, reabsorbed in tubules and broken down by lysosymes. Various chlorinated hydrocarbons (see above) bind to α2μ-globulin and complex persists in tubular epithelium. Lysosomes are damaged, cells die and a cycle of regenerative hyperplasia/necrosis is set up.

THYROID

Chemical goitrogens of various classes, naturally occurring and synthetic

Act mainly by inhibiting synthesis of thyroid hormones (T3, T4) by follicular cells (eg inhibition of thyroid peroxidase, interference with deiodination mechanisms); may also increase degradation and removal of hormones, and inhibit T4-T3 conversion. Low blood levels of T3, T4 provoke sustained increase in circulating TSH which stimulates follicular hyperplasia and development of adenomas and carcinomas. TSH-dependent tumours regress if goitrogenic stimulus is withdrawn. An additional mutational event is usually required for the development of TSH-*independent* carcinomas.

BLADDER

Sodium salts of moderate to weak acids: saccharin, ascorbate, o-phenylphenate (calcium salts, parent acids generally inactive), calculi-inducing agents, nitrilotriacetic acid (NTA)

Transitional cell papillomas or carcinomas in rats; large doses (usually > 1% of the diet) for long periods. Sodium salts listed above cause chronic polyuria. There is compensatory enlargement of the bladder with mucosal hyperplasia. Urinary [Na^+] and pH apparently not directly involved. Failure of urine-concentrating mechanisms in old rats contributes.

High doses of NTA cause loss of Ca^{++} and other cations from urothelium. Cycles of cell death and regenerative hyperplasia follow.

STOMACH

Butylated hydroxyanisole (BHA)

Squamous papillomas and carcinomas in forestomach, mainly in rats especially Sprague-Dawley, Wistar strains. Tumours localized to prefundic margin, following long-standing epithelial hyperplasia. No tumours in adjacent distal oesophagus although dysplastic changes have been noted here in other species treated with BHA.

NOTE Some of the compounds listed here show limited, weak or equivocal evidence of genotoxic activity in various short-term tests.

(Data compiled from Butterworth and Slaga (1987), Cohen *et al* (1991))

the more likely it is that such damage will be incurred. These observations imply that mutations may, at least in certain contexts, occur rather late in the course of tumour development. They also raise the question of the nature of the mutagenic stimulus, given that a non-genotoxic agent is initially involved. Some non-mutagenic compounds may (in part) act as *indirect* genotoxins—for example, releasing H_2O_2 and active oxygen species which can damage DNA by modifying DNA bases and breaking the sugar-phosphate backbone. Alternatively, the genotoxic damage may be inflicted by *endogenous* mutagens which, like their exogenous counterparts, form covalently-bound adducts with DNA. The resulting damage, if imperfectly repaired, is then "fixed" and transmitted to future cell generations as a mutation. Endogenous mutations may occur as a result of oxidative and hydrolytic damage to DNA and are sometimes regarded as spontaneous events, concomitant with tissue ageing. (It is interesting in this context to note that about two-thirds of spontaneous hepatocellular tumours developing in old untreated $B6C3F_1$ mice contain H-*ras* oncogenes. Activated *ras* proto-oncogenes are not, however, regularly found in other spontaneous tumours in this strain nor in spontaneous tumours from rats.)

Non-genotoxic agents may stimulate and derange cell proliferation in other ways which are complex and ill-understood. Examples include disturbances in normal cell-cell communication and alterations in the normal mechanisms which control cell proliferation such as paracrine and autocrine loops.

The effects of non-genotoxic agents on cell proliferation are sometimes described as promotion, and non-genotoxic agents are designated as promoters. Both terms seem inappropriate. Promotion in the original sense was regarded as clonal expansion of initiated cells and it has already been pointed out that for non-genotoxic compounds, cell proliferation probably precedes any mutational event which could be regarded as equivalent to initiation. Furthermore, such proliferation will not be clonal. Secondly, some non-genotoxic agents act in ways other than by stimulating and perverting cell proliferation: immune suppression, for example, may facilitate expression of oncogenic viruses or derange normal surveillance and control mechanisms.

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2. Epidemiology

Epidemiology is the study of the distributions and causes of disease, primarily within human populations. Originally concerned with infectious illnesses, epidemiological investigations are now increasingly applied to non-infectious conditions as diverse as coronary heart disease, schizophrenia and cancer. Epidemiological evidence is extremely useful when evaluating putative human carcinogens because the data are derived directly from human populations and the problems of extrapolating observations from experimental animals are avoided. There are, however, important limitations and sources of potential error in epidemiological studies which are discussed in this chapter. For more detailed accounts, the reader is referred to the preamble to each of the IARC monographs which assesses carcinogenic risk[1] and to a number of basic texts[2–5].

There are 3 main epidemiological approaches to the study of the causes of cancer in man: ecological studies, case control studies and cohort studies. In all of them the intention is to identify differences in risk for one or more specified tumours between different groups in the population or between different populations, and then to determine the extent to which these differences in risk can be attributed causally to specific exposures such as chemicals or other exogenous or endogenous factors.

Ecological studies

Ecological studies, sometimes called correlation studies, involve investigations of populations such as those in a geographical area or within different sets of time periods. Cancer risks in these populations are related to some measure of exposure created from a separate data source. Because information on exposures of interest is rarely available at the individual level, it is difficult to exclude biases such as confounding — exemplified by the observed link between rates of bladder cancer in different populations and the average consumption of coffee. It is not possible to be confident that this is a true observation or, alternatively, that coffee drinking might be a covariate of a known risk factor for bladder cancer such as cigarette smoking. Ecological studies can, however, be useful as exercises which generate hypotheses and, since the measure of risk available is often an absolute measure, they provide a direct indication of the magnitude of a potential public health problem[6].

Analytical studies

Case control and cohort studies approach the evaluation of risk to an individual. Cohort studies are a basic tool of cancer epidemiology and, with their various adaptations, are the preferred means of investigation whenever feasible. A group (or cohort) of individuals, for whom information is available

regarding exposures of interest and other covariates (such as cigarette smoking), is followed forward in time and the development of cancer is documented, usually in terms of death. If the group is identified and exposure recorded in the present and disease occurrence recorded in the future, the study is known as a *prospective* or *concurrent* cohort study. More often, however, attempts are made to use existing records of former exposures to define a group in the past, which is then traced forward in time to the present noting those individuals who have died or developed a defined illness. Such studies are termed *historical* cohort studies. Cohort studies provide measures of the absolute excess risk associated with different exposures. Industrially exposed groups are often used to define a cohort and a specific chemical exposure, and it was by means of cohort studies that 2-naphthylamine and benzidine were identified as bladder carcinogens[7].

An adaptation of the cohort approach has been increasingly used in recent years in which a study population is identified, together with some exposure information, and followed over time. All disease events of interest are registered and detailed information is then sought solely on cases of interest and a sample of the cohort. In this way, resources may be concentrated on the informative individuals. Such a design constitutes a nested case control study. Often, however, cases and controls are not defined rigorously as arising from a specific cohort. Current cases of disease are identified, and individuals chosen from an apparently comparable population free of the disease in question are used as 'controls' or 'referents'; information on past exposure to a suspect carcinogenic factor is obtained retrospectively in the two groups. This design constitutes the retrospective case control study. This approach is commonly used to investigate malignant disease, partly because many tumour types are uncommon so that classic cohort studies—even with many thousands of participants—may only reveal a few examples of a given tumour type. An early and celebrated case-control study was that conducted by Sir Austin Bradford Hill and Sir Richard Doll in the 1950s, which convincingly showed that cigarette smoking was strongly associated with a risk of lung cancer[8].

Rare cancers are unlikely to be studied satisfactorily by the cohort technique unless the associated risk is very large. On the other hand, certain tumours linked to specific carcinogens have been identified just because of their extreme rarity in the general population; examples of 'marker tumours' and their linked environmental risk include hepatic angiosarcomas (vinyl chloride), adenocarcinomas of the paranasal sinuses (wood dust), clear cell adenocarcinomas of the vagina (maternal exposure to diethylstilboestrol) and mesotheliomas (asbestos). In addition, and reflecting the multistep models of carcinogenesis, case-control studies can investigate more than one exposure or putative exposure at a time. In that sense they are directed to understanding a disease, rather than the spectrum of risks associated with a hazardous substance which is best studied by cohorts.

Case-control studies are particularly valuable in testing hypotheses created by clinical observations from case reports or from ecological analyses. They were successfully used, for example, to define the risks of vaginal adenocarcinoma

among young women whose mothers were exposed to diethylstilboestrol during the first trimester of pregnancy[1].

Sources of data used in cancer epidemiological studies

For ecological work the disease data are available either as *mortality* or *morbidity* statistics. Mortality data are regularly published for England and Wales and for Scotland. Morbidity data are generally available through regional cancer registries or as central compilations from the Office of Population Censuses and Surveys for England and Wales. It is not always possible to be sure of the accuracy of these data, but the nature of inference derived from most ecological studies is such that it is usually acceptable. Other information used in epidemiological investigations—for example social indicators, cigarette consumption, chemical or drug use—is derived from a range of sources and its accuracy will understandably vary.

Cohort studies require personal identification details of the individuals entered into the cohort so that their ultimate disease outcomes may be ascertained from the National Health Service Central Registries for England and Wales and for Scotland. Exposure data are important but, inevitably, they vary in quality. At worst they may consist of an occupational history in a particular factory, at best (and very rarely) continued quantitative measures of exposure to a suspect carcinogen may be available.

Case-control studies use cancer registries to be sure that bias from under-ascertainment of cases has not occurred but, by and large, such investigations identify cases at the point of diagnosis from direct clinical contacts. The accuracy of recall is of great importance and any items crucial to the study are, whenever possible, checked against other data sources such as occupational or medical records. Controls are found in a variety of ways—either through Family Health Service records, birth registers or from general practitioner records.

Quality of epidemiological studies: bias, confounding and measurement error

It is necessary to take into account the possible roles of *bias, confounding* and *measurement error* in the interpretation of epidemiological studies. *Bias* is the operation of factors in the design or execution of a study that lead erroneously to a stronger or weaker association between disease and a risk than in fact exists in the underlying population. One major source of error is *selection bias*, whereby cases of disease and the comparison group are chosen differently from the underlying population. For example, a study on a cancer treated at a specialist oncology centre would include cases originating from a wide area and, in such circumstances, it would be erroneous to select controls from people living near a centre of this kind. The local population could well have an entirely different social, age, ethnic and occupational structure compared with that of the wider areas from which the cases were drawn. Another source of error is *recall bias* where information on exposure is obtained by interview in a non-

comparable way from the two groups. It might be argued that patients, because their attention has been focused on their illness, will remember certain past events in a way which differs from that of a member of the general public chosen at random for interview.

Both selection-bias and recall-bias can in principle be eliminated from cohort studies, provided that follow-up is of high quality and the information genuinely obtained before disease status is ascertained. Retrospective case-control investigations are particularly prone to both types of bias, and there needs to be convincing evidence that the study design was adequate to prevent the intrusion of such biases into the results.

Confounding occurs when an association between a disease and an exposure does not represent a true causal relationship. The association, in whole or in part, is the consequence of an apparent link between the putative causal factor and another factor that is associated with either an increase or decrease in the incidence of the disease. Coffee drinking and bladder cancer might, for example, be linked in a case-control study, but there is a close relationship between coffee drinking and cigarette smoking in many populations. Careful analysis is therefore required to take account of the effects of cigarette smoking and the risk of bladder cancer before investigating the putative effects of coffee drinking.

Confounding reflects real relationships in the underlying population and cannot be removed by modifying the design of observational studies. Furthermore, confounding is as much a problem in cohort studies as in case-control studies. Control of confounding is achieved by the statistical techniques of stratification or regression modelling[9,10]. By contrast, the randomisation possible in intervention studies, when it is feasible to undertake them, basically removes confounding by eliminating the association between exposure and other factors.

Errors of measurement usually have more serious consequences in epidemiology than in experimental studies. In the latter, the main consequence is to reduce precision. In the former, errors of measurement will bias the estimates of any real effects of exposure and make the control of confounding more difficult or, in some instances, virtually impossible. Errors may be of two main types: they occur either in measuring the exposure or confounding variables, or in classifying the end-point of the disease. Serious difficulties arise with errors in the measurement or estimation of exposure. Such errors will lead to grave over- or under-estimation of any associated excess risks (or possible protective effects), and they will greatly diminish the degree to which confounding can be removed. Insufficient attention to the problems of measurement error can vitiate the interpretation of a mass of data. To reduce the effects of measurement error, one needs to develop techniques for recording exposure and, in particular, to broaden the use of independent repeated measurements; also to use other methods of detecting exposure such as biological markers—see pages 17 and 23.

The most appropriate setting for this approach is a concurrent cohort study, particularly if biological markers of exposure are envisaged with repeated sampling—a technique which should be used whenever appropriate in future epidemiological studies.

In those epidemiological investigations where observed excess risks are not large (eg relative risks of less than 3), neglect of measurement errors may completely vitiate the interpretation. When large risks are found, for example in relation to cigarette smoking or asbestos exposure, these biases undoubtedly occur but they are less of a problem. But for most cancers, large single risks are likely to have been at least identified if not precisely characterised, and new studies will have to be designed to examine relative risks of less than 3 which carefully address such errors.

In modern epidemiological studies of cancer, one would expect pathological confirmation of the particular tumour types using a standardised system such as the International Classification of Diseases (ICD). There are, however, potential problems here; the tissue diagnoses may be suspect or intrinsically difficult, nomenclature and grouping of existing tumours in ICD is sometimes outmoded or controversial, and variants or new tumour types are still being recognised.

The problem of multiple comparisons

The results of epidemiological studies are often presented as if the only variables included in the investigation were those which *a posteriori* showed association with disease—a criticism which applies mainly to case-control studies. The reader has to distinguish between factors which are part of the main hypothesis motivating the study, and those which were included for less obvious reasons. In particular, beware of a scientific paper describing one single association which clearly originates from a larger study reported elsewhere. Disease associations demonstrated as a result of the test of the hypothesis can be interpreted without consideration of multiple comparisons, but for the other results one or two associations out of a hundred in the study are expected to be significant at the 1% probability level. It is often impossible in some reports to distinguish prime hypotheses, and thus no interpretation of the reported risks can be made—especially if no other reports exist on that topic.

Many investigations present findings for sub-groups of the study population, or for various combinations of exposure. Sub-dividing or combining variables in this way also has the potential for generating a multitude of comparisons but, unless clearly defined at the outset, interpretation is difficult.

In cohort studies one often has the parallel problem that many disease end-points can be assessed—that is, tumours of different histological type developing in several different sites. Unless there are *a priori* grounds for focusing interest on one or a few specific types of tumour, interpretation of the study must reflect the multiplicity of possible comparisons.

Criteria for assessing causality

Dose response

One would expect the strength of a genuine association to increase both with increasing *level* of exposure and with increasing *duration* of exposure. Demonstration of a dose response is an important indication of causality, for example in studies of benzene-exposed workers for increased risk of acute leukaemia[11]. The lack of a dose response argues against, but cannot deny, causality.

Specificity of risk to disease sub-groups

Demonstration that an association is confined to specific sub-categories of disease can be persuasive evidence of causality such as in the links between exposure to vinyl chloride and angiosarcomas of the liver[12].

Strength of association

In general terms, the stronger the association, the more likely one is to consider the association causal. One always has the possibility in case-control studies that selection of patients or choice of the control group may introduce bias; but bias becomes less tenable as an explanation of an observed association as the association strengthens. There remains, for example, little rational argument against a causal link between cigarette smoking and lung cancer[13] but there is much controversy about leukaemia risks from exposure to ethylene oxide because certain studies give a much weaker risk than others[14,15].

Temporal relation of risk to exposure

The relationship of excess risk to time since first exposure, duration of exposure and time since last exposure is of major importance in assessing causality.

For most carcinomas a latent period of at least 10 years can be predicted. When exposure is continuous, there is usually little risk until some 10–15 years after exposure starts; the relative risk then begins to increase and reaches a plateau after 30 years or more. There are, however, exceptions. For radiation-induced leukaemia the risk increases more quickly in younger persons and, among recipients of organ transplants, the risk for some lymphomas can increase strongly within one year[16]. When exposure is short-term or discontinuous, the subsequent evolution of risk may follow a variety of patterns. Work on bladder cancer, for example, shows latency to follow a skewed distribution with modal values at roughly 18 years after first exposure to 2-naphthylamine; a few cases occur earlier, no doubt the consequences of very high exposure or enhanced individual susceptibility.

Lack of alternative explanations

Careful inspection and rejection of all alternative explanations for an association, strengthens the case for a causal association.

Considerations external to the study

It is rare for a single investigation to produce convincing evidence of causality, and corroboration from other studies and evidence of carcinogenicity in

laboratory animals provide additional valuable evidence. Comparison can also be made with the trends in the general population, both in terms of the exposure under study and the distribution of the tumour within the community.

Acceptance of the causal nature of an association would usually require that most of these general criteria were satisfied, together with several corroborating studies and a demonstration of plausible biological mechanisms.

Limitations of cohort studies

In spite of their advantages, the history of cancer epidemiology indicates that cohort studies have not been the major avenue of attack. Case-control studies have predominated. The limitations of cohort studies are summarised below:

i. Prospective cohort studies imply a commitment over many years; both individuals and funding agencies are loath to embark on a project that will not yield its main results for a decade or more. Furthermore, collecting accurate information on more than a short set of variables from the large number of individuals required for a cohort study may be very expensive. The use of case-control comparisons within a cohort may reduce the workload involved in processing the data, but the costs of collecting the data still have to be taken into account.

ii. Historical cohort studies do not suffer from this extended commitment into the future, but they can only be performed if a relevant cohort can be identified. For many substances of interest the existence of such a cohort with accurate records of exposure dating back 10 or more years cannot be guaranteed.

iii. Most cancers are rare. Even taking one of the most frequent examples—mammary carcinoma among women over 50 years of age from western countries—no more than one or two cancer cases would be expected per 1,000 women per year. For most other neoplasms, the expected numbers would be considerably less. In fact, for rare cancers, cohort studies are unlikely to be of much value, unless the relative risk associated with the exposure under study is very large. Given the relative ease with which a series of several hundred cases of cancer at many specific anatomical sites can be assembled, it is clear that on most occasions a comparison of the effort or cost per case included in the study will favour the case-control rather than the cohort approach. In most instances, justification for a cohort study has to be based on the superiority of the information it can yield.

iv. It is difficult to obtain estimates of attributable risk from cohort studies. On many occasions, there is interest not only in the degree of risk associated with a certain exposure, but also the importance of that risk in the general population. For a given cancer site, this can be expressed as the population attributable risk, a quantity which population-based case-control investigation can provide in straightforward fashion. Many cohort studies, however, are based purposely on groups with a much higher prevalence of the relevant exposure than the general population and so cannot give estimates of the population attributable risk.

Advantages of cohort studies over case-control studies

The advantages of cohort compared with case-control studies may be summarised as follows:

i. Because *all* diseases are examined a wider picture is obtained of the health hazards associated with a given exposure.

ii. Recall and selection bias can usually be eliminated. Differences in recall between cases and controls can distort risk estimates derived from case-control studies through introduction of bias and also because precision of recall often differs between cases and controls. Neither problem should be encountered in cohort studies.

Selection bias in case-control studies is often almost impossible to evaluate. If population controls are used, a large proportion of those originally selected may refuse to participate; if hospital controls are used, the choice of which disease categories to include is difficult to resolve, particularly for complex exposures like diet.

Provided that the follow-up mechanisms do not favour particular exposure groups, which can be checked by examining the data, then comparison of the disease experience among different subgroups of the study cohort should be unbiased. The cohort itself, however, will usually be a selected subgroup of the general population, and the disease experiences of the cohort and that of the general population may not be comparable. The best known example of this lack of comparability is the so-called 'healthy worker effect'. The employed population is generally healthier than the nonemployed population of the same age, and the death rates (for many causes) in this group are lower than the corresponding rates in the general population. Cancer death rates appear to suffer less from the healthy worker effect than rates from most other causes, and cancer incidence rates are probably less affected than cancer death rates.

iii. Exposures that are rare in the general population and responsible for only a small proportion of any specific cancer are more easily investigated.

iv. Information on biological parameters before the onset of overt disease can be available in prospective cohort studies which are appropriately designed—for example, the documentation of aflatoxin exposure by serial measurements of albumin-bound aflatoxin B_1.

v. Information obtained retrospectively in a case-control or retrospective cohort may be too inaccurate to be of use. Exposure to aflatoxin, for example, might be estimated by dietary recall combined with tables of aflatoxin levels in common foodstuffs, but one would not expect such estimates to be accurate. One would require measures of exposure obtained by direct observation of aflatoxin intake, either by assaying diet or measuring aflatoxin levels in body fluids[1].

vi. It should be possible to obtain repeated measurements of exposure.

vii. For some purposes, one requires not relative but absolute measures of risk. Cohort studies provide direct estimates of incidence or mortality rates, as opposed to the ratios of rates estimated from case-control studies.

Biological measures of exposure

For many putative carcinogens, there are inherent difficulties in obtaining accurate measures of the quantitative level of exposure. Increasing attention is being paid to methods which assess the degree of uptake of the compound of interest by the individual by assaying levels in body fluids or the degree of binding to macromolecules[17]. Examples include measurement of cotinine in saliva as a measure of exposure to cigarette smoke, morphine metabolites in the urine as a measure of opium intake, binding to albumin as a measure of ochratoxin intake, and the formation of carcinogen-DNA or carcinogen-protein adducts in a variety of tissues as a measure of direct exposure to a suspect compound. The advantages of this approach are the specificity of the measures for both the exposure and the individual, and the potential for quantitative precision. The disadvantage is that the measures usually relate to short-term exposure and inadequately reflect long-term or past exposures.

Another approach is to use genetic abnormalities as an indication of exposure, such as chromosome aberrations, sister chromatid exchanges, mutations in circulating lymphocytes and the formation of micronuclei[18]. Such methods can reflect accumulated long-term or past exposures but they also have limitations. For example, they may indicate exposure to a mutagen but provide no clue to its identity.

Measures of susceptibility

A more direct measure of potential response to specific carcinogens is that seen in the phenotypic expression of certain enzyme systems known to metabolise carcinogens. Thus phenotypes of the cytochrome P-450-dependent mono-oxygenase system may increase susceptibility to lung cancer[19], and the 'slow' phenotype of the enzyme N-acetyltransferase is thought to enhance susceptibility to bladder cancer[20].

Negative epidemiological results

Studies may report the conclusion that no carcinogenic effect was detected. Such a conclusion needs to be assessed in a similar way to that used for positive findings in which the effects of chance, bias, confounding and measurement error need to be evaluated. The last three may have led to a spurious negative result and this possibility needs to be excluded. Certain other features also require attention:

i. The width of the confidence intervals—although the study was apparently negative, what was the most positive finding consistent with the data?

ii. The level of exposure—was it too low for any effect to be expected?

iii. The duration of follow-up after first exposure—was there sufficient time for a carcinogenic effect to declare itself?

It should be noted that the limits of detectability by epidemiological means may be orders of magnitude higher than the likely effects of what are often considered to be 'safe' levels of a carcinogen. A true relative risk of 1.1 would usually be indistinguishable from no effect, but for lung cancer such a value would represent an increase in lifetime risk of 1%. This figure needs to be compared with values for lifetime risk often proposed as 'acceptably low' (see Chapter 7).

A publication bias away from negative or apparently negative results can lead to a spurious 'positive' relationship being accepted when literature reviews are undertaken.

Conclusion

Epidemiology lacks the control over the observational setting and the precision of measurement basic to experimental science. For this reason, its results are often viewed with misgiving. In contrast, however, some epidemiological studies include many more individuals under observation, and for longer periods of time, than could ever be contemplated experimentally. Bioassays in rodents are necessarily carried out at high dose levels and the combined interactive effects of several different exposures are very difficult to investigate.

For this reason, epidemiology is uniquely relevant for the study of human risk from chemical and other carcinogens. Despite the difficulties detailed above interpretation can achieve some degree of scientific rigour if attention is paid to the following aspects whilst persuing relevant literature:

i. The study population, disease (or diseases) and exposure should be well defined. Cases in the study population should have been identified in a way that was independent of the exposure of interest, and exposure assessed in a way that was not related to disease status.

ii. Account should be taken in the study design and analysis of other variables that can influence the risk of disease and may have been related to the exposure of interest. Potential confounding by such variables should have been dealt with either in the design of the study, such as by matching, or in the analysis, by statistical adjustment. In cohort studies, comparisons with local rates of disease may be more appropriate than those with national rates. Internal comparisons of disease frequency among individuals at different levels of exposure should also have been made in the study.

iii. Information should be available on the accuracy of the estimation of exposure and confounding variables and a sensitivity analysis performed to indicate the extent to which inaccuracies of measurement have affected the estimates of effect.

iv. The authors should report the basic data on which the conclusions are founded, even if sophisticated statistical analyses were employed. At the very least, they should quote the numbers of exposed and unexposed cases and controls in a case-control study and the numbers of cases observed and expected in a cohort study. Further tabulations by time since exposure began

and other temporal factors are also important. In a cohort study, data on all cancer sites and all causes of death should be given to avoid the possibility of reporting bias. In a case-control study, the effects of investigated factors other than the exposure of interest should be reported, as well as some rough indication of the number of comparisons made.

v. The statistical methods used to obtain estimates of relevant risk, absolute cancer rates, confidence intervals and significance tests and to adjust for confounding should be clearly stated by the authors. The methods used should preferably have been the generally accepted techniques that have been refined since the mid-1970s. These methods have been reviewed for case-control studies[9] and for cohort studies[10].

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3 Carcinogenic chemicals

As indicated in Chapter 1, it is convenient to divide carcinogenic chemicals into two general classes, genotoxic (reactive towards DNA) and non-genotoxic. The distinction between these categories is sometimes imprecise—for example, in the case of certain non-genotoxins which may exert their effect (at least in part) through secondary production of DNA-damaging species. Compounds in both classes may require metabolism to convert them to active carcinogenic moieties.

Genotoxic chemical carcinogens

The pioneering work of J A and E C Miller[1] established that most genotoxic carcinogens are either electrophilic reagents or are converted to such reagents by metabolism. Electrophiles interact with nucleophilic centres to form covalently bound adducts. Amongst these nucleophilic centres are the DNA bases and phosphates, interaction with which may cause mutation and ultimately cancer.

Many critical chemical groups within a molecule are now known which are involved in genotoxic interactions. Following surveys of 301 chemicals that had been evaluated for carcinogenicity in rats and mice, Ashby and his colleagues[2,3,4] have depicted these genotoxically active groups in a theoretical model compound—see Figure 3.1. The application of such structure-activity relationships is highly successful for qualitative prediction of mutation in bacteria and tumour induction in rodents. Artificial intelligence systems are now also being used with some success for predicting carcinogenic effects from chemical structure and mutagenicity[5].

The mutagenic and carcinogenic potency associated with carcinogen-DNA adducts depend on the chemical nature of the nucleophilic centres where the interaction has occurred. Low molecular weight electrophilic carcinogens, such as methylating and ethylating agents, may be cited as examples. There is evidence from such compounds that adduct formation at oxygen atoms such as the O-6 of guanine or O-4 of thymine, which cause mispairing during subsequent DNA replication, may have the most profound effect on initiation of carcinogenesis. Given this knowledge of the electrophilic reaction profiles of alkylating agents, it becomes feasible to predict an order of carcinogenic potency within a class of compounds.

The isomeric structure of a genotoxic molecule should also be considered prior to structure-activity assessments and carcinogenicity testing. Positional isomers, in which the genotoxically active group is located at different sites in the molecule, may vary greatly in tumorigenic activity. Thus 2-naphthylamine is carcinogenic in several species including man, whereas its isomer,

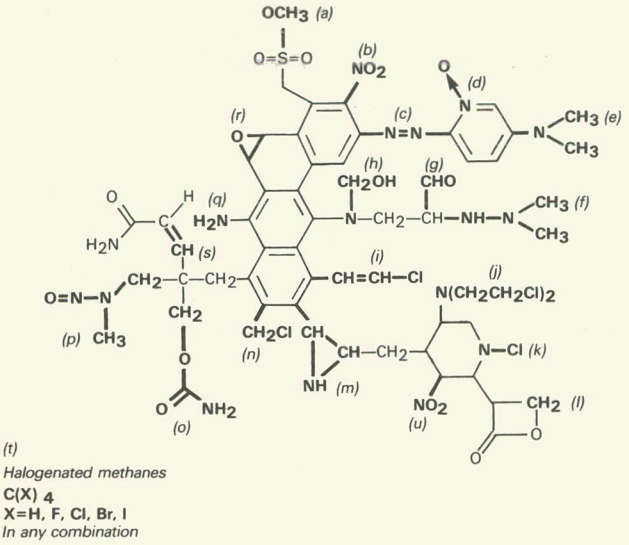

Figure 3.1: Model chemical displaying the key structural features associated with electrophilic (DNA-reactive) carcinogens.

The basis of the structure has been discussed in detail by Ashby *et al*[2,3,4]. Some substituents are naturally electrophilic [eg a)] while others require metabolic activation in order to produce an electrophile [eg b)]. The level of evidence varies between the sub stituents. For example, a wealth of evidence supports a potential genotoxic hazard from aromatic amino groups [q)]. In contrast, the chloramine group [k)] is unique among those shown in being associated with genotoxicity but not yet carcinogenicity. Halogenated alkanes [t)] are a large group that contains some mutagens and some carcinogens, the requirements for activity being obscure at present. Substructure (s) represents Michael condensation reactive chemicals as evidenced by acrylamide.

1-naphthylamine, has not been shown to induce tumours in animals—probably because the processes of metabolic activation required for aromatic amines to become genotoxic are not effective for 1-naphthylamine.

Optical isomers may also differ in their genotoxic effects. This phenomenon is particularly apparent for the active metabolites of polycyclic aromatic hydrocarbons (see page 25). Thus for benzo[a]pyrene it is believed that a 7,8-dihydrodiol-9,10-epoxide is responsible for the activity of the hydrocarbon. Out of the four possible stereoisomers that could be derived from a trans 7,8-dihydrodiol, only one shows pronounced tumorigenic activity[6]. (This stereoisomer is also the predominant one formed by metabolism of benzo[a]pyrene). It is therefore apparent that compounds being tested for carcinogenicity should be of a known degree of isomeric purity.

Genotoxic carcinogens also interact with nucleophiles other than DNA such as proteins or glutathione, either spontaneously or through enzyme mediation. Reaction at these sites may generally be regarded as protection against carcinogenic effects although there are a few chemicals, notably some halogenated hydrocarbons, where reaction with sulphydryl groups (as in glutathione) results in chemical activation of the genotoxic species. For example, the

interaction of glutathione with 1,2-dichloroethane, catalysed by glutathione transferase, yields the S-(2-chloroethyl) derivative of gluthathione. This compound, which has a structure analogous to a sulphur mustard, is a powerful alkylating agent. The extent of such activiation will depend on the presence of the appropriate glutathione transferase isoenzyme, levels of which vary in different species.

For active electrophilic genotoxic compounds, reaction also occurs with water (which is weakly nucleophilic), resulting in detoxified hydrolysis products. Attention should be paid to this possibility in the design of dosing schedules when testing such compounds for carcinogenic activity.

The quantitative determination of carcinogen-nucleic acid adducts in an exposed individual may give an indication of the biologically-effective dose of the compound received (see Chapter 2). Proteins may also be used for adduct determination, since it has been shown for a variety of compounds that the extent of protein-adduct formation relates quantitatively to the extent of nucleic acid-adduct formation. However, it is important to establish this relationship within each series of compounds before this approach can be adopted. The use of proteins has the advantage that they are more readily accessible in quantity from man than nucleic acids. Haemoglobin is very suitable for such studies: it is abundant in blood and has a life-span of 4 months in man, thereby allowing assessment of carcinogen-exposure to be carried out considerably later than the actual exposure. Nucleic acids for studies of adduct determination are generally also isolated from blood samples, although placenta and tissue explants have been used. The analytical methods required to detect carcinogen adducts with proteins or nucleic acids in man have to be exceptionally sensitive and include immunoassay, mass spectrometry and [^{32} P]-post-labelling procedures. When fully developed, biological monitoring techniques of this kind[7,8] should have great potential for identifying genotoxic compounds to which man is exposed and determining the doses of each compound received by individuals. They have a useful role in hazard assessment but there are many problems in their application to human risk assessment. For the latter, epidemiology will always provide the most valuable information (see Chapter 7).

Metabolism

In addition to the structure-activity relationships described above, the carcinogenic potency of a compound is also governed by the amount of genotoxically-active species that reaches the target DNA *in vivo*. Many separate phases are involved including whole-body distribution of the chemical and its subsequent metabolism, generating either an activated or a detoxified species.

One of the most important metabolising systems for carcinogens is cytochrome P-450-dependent mono-oxygenase which exists not as a single enzyme but as a family of isoenzymes. Complementary DNAs (cDNAs) have been isolated and sequenced for many cytochrome P-450 variants. Distinct P-450 gene families have diverged during evolution from a common ancestor; 78 unique P-450

genes divided into 14 families have now been identified[9]. Cytochrome P-450 isoenzymes may be tissue-, species- or substrate-specific so that extrapolation of carcinogenicity from species to species for compounds that are activated by metabolism is often very difficult. Among the activation processes catalysed by cytochrome P-450 are epoxidations of olefins, aromatic hydrocarbons, vinyl chloride and aflatoxin B_1; N-oxidation of aromatic amines and amides; and hydroxylation of alkyl nitrosamines[10].

Metabolic activation of carcinogens is not, however, exclusively associated with cytochrome P-450. Other activating systems include prostaglandin synthetases which produce reactive species from diethylstilboestrol; bacterial glycosidic enzymes which hydrolyse the plant carcinogen cycasin to the methylating agent methylazoxymethanol; and DT-diaphorase which is involved in the reduction of 4-nitroquinoline-N-oxide to an active hydroxylamine.

Metabolic detoxification of genotoxic metabolites occurs through the action of a variety of enzymes, including epoxide hydrolase and the conjugating enzymes which couple xenobiotic metabolites with glutathione, glucuronic acid, sulphate, phosphate or acetate. Conjugates are generally excreted relatively intact, although in rare instances they can act as transport forms for the active metabolite if a suitable deconjugating system is present in the target tissue. Furthermore, some glutathione conjugates are active in their own right and react with nucleophiles before they can be excreted.

The enzyme responsible for glutathione conjugate formation, glutathione transferase, also exists in numerous isoenzymic forms which may result in important interspecies differences in carcinogenicity. Thus in the case of aflatoxin B_1 (AFB_1), the most active metabolite is the 8,9-epoxide which may be detoxified by glutathione transferase. Mice are less susceptible to the carcinogenic effects of AFB_1 than rats, partly because their livers are much more efficient at conjugating the AFB_1-epoxide with glutathione.

Of particular importance in the assessment of human risk associated with exposure to genotoxic carcinogens has been the recent discovery of genetic variation (polymorphism) in the metabolism of some classes of genotoxic compounds such as aromatic amines and aryl hydrocarbons. For aromatic hydrocarbons there is a 40-fold range among normal subjects in the metabolic capacity of aryl hydrocarbon hydroxylase (AHH) to produce genotoxic metabolites from these compounds. Correlations have been drawn between high AHH activity and lung cancer incidence[11]. In the case of aromatic amines, there is polymorphism in a deactivation process (N-acetylation), which distinguishes two groups of individuals of different hypothetical susceptibility to tumour induction by aromatic amines.

Examples of genotoxic chemical carcinogens

The examples that follow are not intended to give a comprehensive survey of genotoxic chemicals, but the mechanisms discussed are representative of most genotoxic carcinogens.

Direct-acting genotoxic agents

This group comprises a diverse range of reactive chemicals which do not require metabolic activation in order to exert their carcinogenic effects. Generally known as alkylating agents, they include epoxides, imines, small-ring lactones, sulphate or sulphonate esters, phosphate esters, active alkyl halides, nitrosoureas and nitrosamides. Some of them occur widely in the environment and in occupational surroundings. Many anticancer drugs such as 1,4-butanediol dimethane sulphonate (myleran) and the nitrogen mustards are alkylating agents and their carcinogenic effects are well known. The mutagenic/carcinogenic potency of these compounds may be related to their ability to react with specific sites in DNA: the methylating agent N-methyl-N-nitrosourea, for example, shows a higher proportion of O–6 alkylation of guanine compared with methyl methanesulphonate or dimethyl sulphate and is a more potent carcinogen in animal systems[12]. Increased O–6 alkylation of guanine is observed for compounds that react by way of a unimolecular substitution mechanism rather than a bimolecular mechanism.

Activation-dependent genotoxic agents

Polycyclic aromatic hydrocarbons

These carcinogens, the first to be chemically characterised, are abundant at low levels in the environment through their formation by incomplete combustion of organic material. Much higher exposures occur in cigarette smokers. Polycyclic aromatic hydrocarbons are metabolically activated via formation of an electrophilic epoxide: the most extensively studied example is benzo[a]pyrene which yields a 7,8-dihydrodiol-9,10-epoxide as the ultimate carcinogenic metabolite. The metabolite then reacts with nucleophilic sites in DNA by means of an active electrophilic centre adjacent to the so-called 'bay-region' of the molecule. Generation of active bay-region diol epoxide seems to be applicable for many polycyclic aromatic hydrocarbons. Carcinogenic activity is less for those compounds which, on metabolism, yield less bay-region diol epoxide or which do not possess a bay-region. Genotoxic activation by epoxidation of double bonds also occurs for many structurally simpler chemicals such as styrene and ethylene.

Aromatic amines

Human exposure to aromatic amines may occur from sources such as industrial chemicals and tobacco smoke. It has been demonstrated that powerfully mutagenic heterocyclic amines are produced in small amounts during pyrolysis or cooking of protein-containing food. The major metabolic activation process for aromatic amines appears to be N-hydroxylation. The hydroxylamine that is produced either interacts directly with DNA via an electrophilic nitrenium ion or is conjugated to form a reactive electrophilic ester such as sulphate. Similar activation processes occur for aromatic amides and for nitroaromatic compounds. The carcinogenic potency of aromatic amines will therefore depend in part on the extent of metabolism to the hydroxylamine. Another important metabolic pathway for aromatic amines is N-acetylation, a detoxification process which has a polymorphic distribution in man. Individuals who

are slow acetylators may theoretically have an increased risk of developing certain forms of cancer, whereas fast acetylators could be protected.

Nitrosamines

Many alkyl nitrosamines are carcinogenic in experimental animals. Man is exposed to nitrosamines either as preformed compounds or through the nitrosation of amines by nitrite under the acidic conditions of the normal stomach. Nitrosamines are metabolically activated by hydroxylation at the carbon adjacent to the nitrosamine group; spontaneous decomposition follows with formation of an aldehyde and an electrophilic alkyldiazonium ion which interacts with DNA. As with the polycyclic aromatic hydrocarbons, the extent of initiation of cancer from nitrosamines will depend on the extent of metabolic activation which in turn will vary according to the compound involved, the amount and degree of induction of the relevant activating enzyme, and the presence and concentration of modulators such as vitamin C and α-tocopherol.

Other naturally occurring carcinogens

Several species of microorganisms and plants produce genotoxic carcinogens. Some of the best documented examples are fungal metabolites such as the mycotoxin aflatoxin B_1 which is a powerful hepatocarcinogen in many species. It is metabolically activated by oxidation of a double bond in its furan ring, yielding a DNA-reactive electrophilic species. Carcinogenic products from plants include alkenyl-benzenes such as safrole and cycasin, and the pyrrolizidine alkaloids; all of them are metabolized to reactive alkylating species.

Inorganic carcinogens

Various chromate salts (Cr(VI)) are recognised as human carcinogens. The genotoxic effects of Cr(VI) may be due to its reduction in the cell to Cr(III) which binds to nucleic acids. Other inorganic chemicals which have been implicated as carcinogens are certain salts of arsenic, nickel, beryllium and lead. Their mechanism of action is still unclear and may be different from the electrophilic interaction described here for organic chemicals. Effects on DNA polymerases which result in infidelity in DNA synthesis have, however, been observed. Cisplatin [*cis*-dichlorodiammine platinum(II) dichloride] is a cytotoxic drug which does interact directly with the nucleic acid and is carcinogenic.

Non-genotoxic chemical carcinogens

Non-genotoxic carcinogens are believed to exert their carcinogenic effects through processes which do not involve direct binding of the chemical or its metabolites to DNA (see Chapter 1). The biochemical modes of action of non-genotoxic carcinogens are diverse and, for the most part, are very poorly understood.

Inducers of the cytochrome P-450 enzyme system act as non-genotoxic carcinogens, principally in the liver of mice and (to a lesser extent) rats. Examples include a number of halogenated hydrocarbons and their oxygen-containing derivatives, polychlorinated biphenyls, phenobarbital, dioxin and

peroxisome proliferators such as the hypolipidaemic drugs clofibrate and fenibrate and the plasticiser di(2-ethylhexyl)phthalate.

Peroxisome proliferators are thought to induce one cytochrome P-450 isoenzyme resulting in an increase in ω-hydroxylation of fatty acids which are then further oxidized to long chain dicarboxylic acids. Peroxisomes subsequently proliferate to convert the increased amounts of these acids to chain-shortened fatty acids. It was suggested some years ago that induction of the peroxisomal fatty acid β-oxidation systems might liberate H_2O_2 and/or active oxygen species which damage DNA; a measure of protection against such damage would be produced by peroxisomal catalase and glutathione peroxidase. Peroxisome proliferators might thus act, at least in part, as indirect genotoxic agents. Supporting evidence is, however, scanty and an alternative view is that peroxisome proliferators disturb the normal processes of cellular growth control by inducing sustained liver cell hyperplasia and enhanced cell turnover[13]. There are species variations in the P-450 induction caused by peroxisome proliferators and in the levels of the protective enzymes which, in turn, are reflected by wide interspecies differences in susceptibility to the hepatocarcinogenic effects of these agents. The capacity of hepatic peroxisomes to proliferate is intense in rats and mice but negligible or absent in most other species, including primates and man.

Phorbol esters such as 12-0-tetradecanoylphorbol-13-acetate (TPA) have been widely studied as promoters for carcinogenesis in mouse skin although TPA is, under certain circumstances, a complete carcinogen in its own right. TPA stimulates proliferation and clonal expansion in initiated cells, operating in contexts of chronic tissue damage and repair. The biochemical effects of TPA include enzyme induction (particularly of ornithine decarboxylase but also of 5′-nucleotidase, ATPase and the plasminogen activator system), alterations in membrane function (changes in Na^+/H^+ fluxes, phosphorylation of cell surface receptors for growth factors and other proteins), and generation of free oxygen radicals. Most of these effects appear to be associated with activation of a calcium^{++}-dependent, cAMP-independent protein kinase C which has now been identified as the receptor for phorbol ester tumour promoters[14].

Hormones. Chronic perturbation of the endocrine system may result in tumours in the appropriate target tissues and (sometimes) the liver. Some hormones and hormone-modifying compounds may, however, act in other ways. The herbicide 3-amino-1,2,4-triazole, for example, is a promoter for thyroid follicular tumours by virtue of a direct antithyroid effect mediated through inhibition of peroxidase-independent iodide oxidation within the gland parenchyma; but it may also be genotoxic by virtue of the macromolecular binding which results from activation by peroxidative metabolism. The synthetic oestrogen compound diethylstilboestrol gives rise to a genotoxic metabolite, the 4-4′-quinone, which binds to DNA both *in vitro* and *in vivo*; again the relative contributions of hormonal and genotoxic effects are unclear.

Physical carcinogens. Examples include implanted plastics and various naturally occurring and artificial fibres, notably asbestos. Such substances are

chemically inert: no structure/activity relationships can be derived and their mode of action is related to their physical properties. Asbestos may provide an example of a carcinogen which exerts both genotoxic and non-genotoxic effects. The fibres damage chromosomes, induce aneuploidy and are intensely cytotoxic to mesothelial tissues in which they set up repeated cycles of cell death and cell regeneration.

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4. Use of short-term tests

General considerations

Current understanding of the mechanisms whereby tumours develop (see Chapter 1) indicates that any substance that induces mutations in mammalian cells must fall under the suspicion of being carcinogenic. Many compounds shown to be mutagenic in whole animals have not been tested for carcinogenicity but, of those that have, the vast majority are carcinogenic in laboratory rodents. It therefore seems reasonable and prudent to include results from short-term mutagenicity tests in any assessment of putative carcinogenicity and, in particular, to regard compounds which are mammalian mutagens as having carcinogenic potential even though such potential has not been examined or has not been demonstrated in formal carcinogenicity tests. There are a very small number of chemicals which clearly induce *in vivo* mutations in mammals but which are not detectably carcinogenic in standard long-term bioassays. The reasons for this discrepancy are unclear: the carcinogenic process cannot be viewed solely in terms of mutagenic events (Chapter 1) but, equally clearly, conventional long-term bioassays have definite limits in sensitivity (Chapter 6).

The expression of carcinogenic potential sometimes varies markedly between species, often (at least in part) because of differences in absorption, distribution and metabolism of the substances under consideration. The results of mutagenicity tests shed little light on these specific processes; and they can rarely be used to predict which species (if any) will be susceptible to tumour induction. Nor do they give quantitative information about the carcinogenic potency of a chemical, nor the target issues in which it may act.

Numerous agents are carcinogenic to animals but, despite extensive testing, some show no detectable mutagenic or clastogenic activity. Such compounds are described as non-genotoxic carcinogens (see Chapters 1 and 3). Negative results from a reasonable set of well conducted mutagenicity assays cannot, therefore, be construed as proof that a compound is non-carcinogenic. There is, unfortunately, no comprehensive and generally accepted panel of short-term tests for the detection of non-genotoxic carcinogens. Therefore in circumstances where human exposure to such substances is likely to be extensive (in terms of amount and/or duration) there is, at the present time, no acceptable alternative to long-term carcinogenicity studies in rodents.

The genetic changes involved in carcinogenesis are many and varied[1] (see Chapter 1), and future tests may be developed with genetic end-points more closely related to specific stages in the carcinogenic process. At present, however, simple well-validated mutational end-points are recommended and the current philosophy is to require information on the three levels of mutation: gene, chromosome (structural) and chromosome (numerical, eg aneuploidy).

Short term mutagenicity tests may be deployed in various ways:

i. to demonstrate that a substance has significant *in vivo* mutagenicity and thus may be assumed to have carcinogenic potential.

ii. to determine priorities for long-term carcinogenicity testing. The need for long term animal studies is unlikely to be justifiable in cases where *in vivo* mutagenic activity has been demonstrated, it being assumed that the compound has carcinogenic potential. There may be cases where mutagenic potential has been demonstrated *in vitro* and where it is not possible to carry out appropriate *in vivo* mutagenicity studies; the demonstration of lack of carcinogenic potential for such compounds in chronic animal studies is particularly valuable.

iii. to compare, in certain instances, the relative mutagenic potency of a chemically related group of substances. In some cases this may also be reflected in carcinogenic potency but such a correlation is by no means universal.

iv. to aid in the interpretation of problematic results of long-term carcinogenicity assays.

There is an increasing tendency for negative results in such mutagenicity tests to be interpreted (in the absence of other data) as reflecting lack of genotoxic carcinogenic potential which, in the case of substances with low-level human exposure, may be tantamount to regarding them as non-carcinogenic for practical purposes. This assumption is not totally justifiable because certain non-genotoxic carcinogens are known which are active at low concentrations: 2,3,7,8,-tetrachlorodibenzo-*p*-dioxin (TCDD) may be cited as an example. It does, however, serve to emphasise the fact that practicable short-term methods for the detection of non-genotoxic carcinogens are not yet available, and that resources for long-term animal bioassays are inadequate for the examination of all substances with low-level human exposure even if their use were to be considered justified.

A more detailed discussion of all aspects of mutagenicity testing and its implications for carcinogenicity may be found in the Guidelines for the Testing of Chemicals for Mutagenicity[2].

A strategy for mutagenicity testing in the context of carcinogenicity

It is not justified to use whole animal mutagenicity tests for general screening purposes since a set of three *in vitro* tests, conducted according to protocols, should detect virtually all compounds with mutagenic potential. Only in circumstances where humans are likely to be exposed to high levels of a substance or to moderate levels for prolonged periods (for example, certain therapeutic drugs and food additives) or where chemical structure or other features of the test compound trigger structural or other alerts (see Chapter 3) will additional *in vivo* testing be necessary.

The mutagenic potential of a substance should therefore be investigated using *in vitro* tests, each of which should be repeated at least once. Two tests are recommended: a bacterial assay for gene mutation and a test for clastogenicity in cultured mammalian cells. Where human exposure would be expected to be extensive and/or sustained, and difficult to avoid, a further test for gene mutation in mammalian cells should be carried out.

If mutagenic activity is demonstrated reproducibly in any *in vitro* test, then it is necessary to investigate whether this potential is also expressed *in vivo*. The recommended assay is for the production of chromosome damage in bone marrow cells using either metaphase or micronucleus analysis. Routes of exposure that are unlikely to give rise to significant absorption in the test animal should be avoided in the first instance although there may be a case for follow-up studies using a route of administration that reflects exposure conditions likely to occur in use.

Figure 4.1: Testing strategy for investigating the mutagenic properties of a substance in the course of assessment for carcinogenic potential[2].

STAGE 1

Initial Screening

Two tests required (a + b) except where human exposure would be expected to be extensive and/or sustained, and, difficult to avoid, when all three tests are necessary

In Vitro Tests

(a) Bacterial assay for gene mutation

(b) Test for clastogenicity in mammalian cells (for example metaphase analysis)

(c) Test for gene mutation in mammalian cells (for example the L5178Y TK + / − assay)

STAGE 2

Tests for:
Compounds positive in one or more tests in Stage 1,
and
All compounds where high, or moderate, prolonged levels of human exposure are anticipated

In Vivo Tests

(a) Bone marrow assay for chromosome damage (metaphase analysis or micronucleus test)

Plus, if above negative, and any *in vitro* test positive.

(b) Test(s) to examine whether mutagenicity or evidence of DNA damage can be demonstrated in other organs (eg liver, gut etc)

Where there is unequivocal evidence for mutagenicity in one or more of the *in vitro* tests and if the *in vivo* assay for chromosome damage in the bone marrow is negative, then further investigations will be necessary if no long-term carcinogenicity assays are to be performed. This is a difficult area and some possible approaches have been discussed in the Mutagenicity Guidelines. Tests for mutagenicity or evidence of DNA damage in other organs such as liver or gut may be needed. There is a clear need for more *in vivo* test systems which use a greater range of target tissues. Meanwhile, data from adequate studies on absorption, distribution, metabolism and excretion of the test substance will be particularly valuable at this stage.

An outline of the recommended testing strategy is given in Figure 4.1: it must not be regarded as an invariable sequence of testing which has to be followed rigidly in all cases. A flexible approach should be adopted with each substance being considered individually, taking a number of factors into account—the chemical structure of the compound and the reactivity of the parent substance and/or its metabolites; information on absorption, metabolism and distribution; any existing knowledge of its mutagenic properties; and its intended use and predicted human exposure.

The need to carry out all studies to a high quality and to meet rigorous, well-validated protocols is stressed. The size of a study should be adequate to obtain significant results. There is therefore a preference for a limited number of studies carried out to a very high standard.

Cell transformation

In addition to the mutagenicity tests discussed above, a number of mammalian cell transformation assays have been developed, based on measuring the ability of a substance to induce morphological changes in cells or colonies in cell culture.

Ever since the first demonstration by Berwald & Sachs in 1965[3] that cells in culture could form morphologically transformed 'foci' when exposed to carcinogenic chemicals, attempts have been made to develop reproducible cell transformation assays which would provide a quantitative assessment of carcinogenic potency. While some measure of success has been attained using rodent cell systems, primary cultures of human cells have proved to be almost completely refractory to transformation *in vitro* using physical or chemical agents. The difficulties stem from the fact that transformation to malignancy involves several discrete stages both *in vitro* and *in vivo*, and involves mutational events which lead to both oncogene activation and functional loss of tumour suppression (see Chapter 1). These processes take many years before malignant tumours appear *in vivo*, and it is in retrospect naive to assume that short-term *in-vitro* tests will encompass all the complexities of tumour development.

Cell transformation *in-vitro* can be sub-divided into a series of steps: acquisition of an indefinite life span (immortalisation), morphological transformation

and development of tumour-forming ability on injection into animals. It would therefore seem logical to assess separately the ability of carcinogenic agents to induce these different end-points. Immortalisation is extremely difficult to achieve by treating primary cultures of human cells with a variety of potent carcinogens, although some success has been obtained by transfection of genes encoded by certain DNA tumour viruses. Immortalisation is much more frequently observed in cultured rodent cells, and attempts have been made to quantify the immortalising potency of various chemicals. The relevance of such assays to human carcinogenesis is, however, difficult to assess because of the major discrepancies in the frequencies with which human and rodent cells develop a transformed phenotype.

Assays are presently being developed for the subsequent stages of *in vitro* transformation by utilising human cells previously immortalised by infection with DNA viruses. These immortalised cells can undergo morphological transformation or even malignant progression by treatment with chemicals or by oncogene transfection. Such assays may eventually be useful in evaluation of this phase of the carcinogenic process.

The most widely used *in vitro* cell transformation assays involve the induction of foci in monolayer cultures of immortalised fibroblasts such as NIH/3T3 cells or C3H10t½ cells. The latter system in particular has a low background rate of 'spontaneous' transformation and has been used to measure the transforming ability of agents as diverse as X-rays, polycyclic aromatic hydrocarbons and certain tumour promoters. One major problem with the use of this focus assay is that the nature of the genetic event(s) induced by the carcinogen has not been elucidated.

Specific molecular changes have now been detected in certain human cancers, including activating point mutations in *ras* genes, and deletions or inactivating mutations in Rb, p53, or DCC genes[4]. It is therefore possible to design target cells harbouring marker genes which can be activated (or inactivated) by molecular mechanisms which are known to be relevant to tumour development. It is probable that such target cells will eventually be used to complement *in vitro* transformation assays to provide more accurate estimates of the carcinogenic potential of chemical agents in mammals.

At the present time it is not clear how the cell changes observed in cell transformation assays relate to the overall carcinogenic process. Furthermore, technical aspects of these assays need to be improved in terms of reproducibility and robustness. It is, however, possible that versions of cell transformation assays will eventually be included among generally accepted short-term tests.

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5. Design of carcinogenicity tests

Carcinogenicity tests require a major commitment of skilled staff, animals and laboratory resources, so it is essential that they are conducted according to the best design and using the most effective procedures. The protocol must take into account scientific concerns, regulatory needs and animal welfare; and the procedures must be adapted to the properties and uses of each compound, particularly to the special problems posed by long-term studies in large groups of animals.

The basic features of conventional carcinogenicity testing are now broadly agreed[1-8]. The general approach described in these sources should be followed as far as possible in accordance with the internationally agreed test methods, which represent the scientific consensus on carcinogenicity testing. This will aid interpretation of the results and will increase their suitability for regulatory purposes; in particular, current findings can be related to past experience. Furthermore this approach will minimise the wasteful and unjustifiable duplication of studies for different regulatory authorities. In this regard the OECD guidelines are important since, under the OECD Council agreement of 1981 on mutual acceptance of data[9], all member countries agreed to accept data on chemicals that were obtained according to an OECD test method and in compliance with the OECD principles of GLP (Good Laboratory Practice)[10].

Carcinogenicity testing is based on a comparison of the incidence, nature and time of occurrence of neoplasms in treated animals and in controls. The conclusion that a substance has had a carcinogenic effect depends on showing that the test substance has materially increased the incidence of neoplasms, made them appear earlier in life or produced unusual types of cancers in treated animals, all in comparison with the spontaneous tumours found in concurrent controls. Behind this apparently simple statement lies a very complex set of requirements and procedures.

The main scientific and technical points to be considered in the design and conduct of carcinogenicity tests are listed in Table 5.1, and the major issues are discussed below. General aspects are presented first, followed by specific points. Both are considered from strictly practical viewpoints, covering the decisions the toxicologist must make in designing the experiment and what information should be provided to explain the basis for them.

Table 5.1: *Main Points of Concern in Designing Carcinogenicity Tests*

Point of Concern	Comment
Toxicokinetics	choice of species and dose levels need for assessment during experiment
Toxicity	importance of pharmacological and toxic effects
Design and analysis	expert advice regulatory requirements
Animals	selection husbandry
Test substance	specification formulation
Controls and dose group	numbers dose levels comparator compounds concurrent and historical controls
Route of exposure	oral or inhalation preferred for systemic effects
Duration of exposure	start early in maturity
Conduct of the experiment	scientific, husbandry, GLP
Observations	in life after death
Pathological procedures	dose groups and tissues to examine nomenclature
Resources, Organisation Record keeping Reporting Safety	adequacy adequacy GLP timeliness

Critical issues in the design of a carcinogenicity test

Toxicokinetics
(See also Chapter 3 'Carcinogenic Chemicals' and the Section on 'Controls, dose groups and doses')

It is highly desirable that the toxicokinetics of the test material are adequately understood *before* carcinogenicity experiments are designed. The animal species used should as far as possible be selected for similarity of the distribution and metabolism of the test compound in the chosen species to what is known about man. Given the limited number of species and strains suitable for carcinogenicity testing (see page 38), the choice will be limited. For each compound the toxicologist should know as much as possible about the comparative rates and extent of absorption, distribution, metabolism and excretion in man and in experimental animals treated by the likely route of human exposure and with the preparation to which man may be exposed. Studies in animals must include examination of multidose kinetics.

The aim as far as possible is to ensure an exposure such that the effects both of the parent substance and its major active metabolites are investigated, including those produced by site-specific processes, such as metabolism in the skin, lung and liver. These considerations are particularly important when testing inactive precursor substances, such as a 'prodrug'. It may be difficult to mimic human exposure precisely and it may, on occasion, be necessary to alter the frequency and magnitude of dosing—for example, by giving the test compound as single or twice daily doses by gavage instead of in the diet or drinking water. Alternatively, the use of a vehicle that enhances or impedes absorption may be considered. The measurement of blood/plasma levels will give evidence of systemic exposure and may allow a comparison to be made between the exposure of the test animals and levels seen in man. If treatment in the experiment is to be by a route which creates a high local concentration, such as inhalation or application to the skin, it may be valuable to measure both the concentration at the site of action (eg the lung in an inhalation study) and systemic exposure to ensure that potential local and systemic effects in humans are adequately explored.

Various important results may accrue from preliminary multidose kinetics and metabolism studies, notably the demonstration of a plateau in absorption of oral doses or the occurrence of non-linear kinetics, which will result in exposure to an abnormally high level of a compound or its metabolites produced by an aberrant pathway. Above all, these studies will provide a sound basis for choosing dose levels for the subsequent carcinogenicity bioassay which will result in adequate exposure of the putative target tissues to the active compound. It is good practice in the formal carcinogenicity test to measure at intervals (at least) levels of the test compound and/or its major metabolites in blood or urine in order to document absorption and to indicate any major change in kinetics due to age or other effects. This information may be useful in explaining interspecies differences of effect. For complex mixtures it may be helpful to analyse for a characteristic marker component as an index of systemic exposure and which can be followed in kinetic studies.

The study of foodstuffs and natural products poses serious problems in terms of toxicokinetics. A 'marker' component might be measured as a surrogate, if its handling were considered representative of the very complex mixture administered, but it may not be possible to examine the overall kinetics. It may be more convenient to make these measurements in an additional subset of animals in the main study rather than by enlarging the group size; it is recommended that they should be made on groups of not less than 3 animals at intervals of 3–4 months.

For compounds with optical or geometrical isomers, or those from which isomers may be generated in the body, it is important to examine the extent of racemisation and isomerisation during the test, again in parallel with investigations in man (see also Chapter 3).

Toxicity

Obtaining the most useful information from a carcinogenicity study might appear to require maximisation of exposure to the test material, but there is need for caution in administering high dose levels (whether systemic or topical) in order to avoid excessive toxic effects or inappropriate toxicokinetics (eg overwhelming detoxification or absorption mechanisms) that would jeopardise interpretation of the results for man. Sustained tissue damage and repair, for example, may enhance tumour development at the affected site by means that are probably irrelevant to man and, in tissues such as the liver, may alter the metabolism and kinetics of the test compound as well. Exposure of this kind should be avoided. Such a policy does not mean that the conventional 'Maximum Tolerated Dose' (MTD) ought to be discarded—just that sufficient understanding of the toxicity of the test material is required to decide what criteria define the MTD, including any reasons for use of a lower dose that might give results more relevant to man. Certain treatment-related effects in target organs, such as hypertrophy or hyperplasia, should be regarded in the first instance as acceptable in carcinogenicity testing, and their occurrence cannot necessarily be adduced as an argument to reduce the dose. Similarly, some toxic effects should also be accepted, unless they become excessive or distressing. Further discussion of the issue of selection of dose levels will be found in the section on 'Controls, dose groups and doses'. Once again, preliminary studies with the test compound—to identify likely target organs and at least the early toxic effects that may be expected—provide information which is necessary before the carcinogenicity test can be adequately designed.

Animal husbandry

Carcinogenicity experiments are of necessity very protracted, and it is essential that high standards of care are maintained and that animal welfare is assured. General advice is given in publications by the Home Office[11] and LASA/UFAW[12–14]. The arguments about group or single housing, variously based on welfare and practical considerations, remain unresolved.

Choice of animals

Experience, practicality and availability determine that tests are conventionally done in any two of three species—mouse, rat and hamster. There would have to be very compelling scientific reason or regulatory demand for others to be employed; those for which there is some background laboratory knowledge (such as the dog, pig, guinea pig, rabbit or a primate) variously entail problems of ethics, size, and the need to dose and follow for many years. A possible case for using such species might, however, be based on a particular metabolic or toxic response to a test compound, which was similar to that of man.

The selection of *strain* is more difficult. There are general arguments in favour both of outbred and inbred animals, and for and against particular strains. The present consensus favours working with well-validated strains, including Sprague-Dawley, Wistar and Fischer (F344) rats, and outbred CD-1 Swiss and hybrid B6C3F1 mice; hamsters used in carcinogenicity studies are usually outbred. The toxicologist must have a sound understanding of the normal

biology of the test species and strains, together with a detailed knowledge of their incidence of spontaneous tumours and other non-neoplastic ailments. The source and full identity of the animals used should always be stated in the final report.

Health status

It is not possible to specify a particular health standard because none exists. The use of 'SPF' (specific pathogen free) animals is valuable, especially in inhalation studies, in view of the importance of avoiding respiratory disease. Such animals carry the need for monitoring and barrier maintenance. Conventional colonies may be successfully used. It is important to document and diagnose all endemic and incidental infections by any type of organism, and to consider whether such infections, or their treatment, might affect the outcome of the test. Sentinel animals, not belonging to the main test group, are often valuable in monitoring infection.

Diet

Animals are generally given unrestricted access to a standard laboratory diet and drinking water, both of which conform to accepted international standards and the requirements of GLP to demonstrate quality and the absence of harmful contaminants.

There is evidence that the high and variable incidence of certain types of neoplasms in untreated control animals may be due at least in part to the custom of freely providing a diet of excessively high nutritional quality. The incidence in rats and mice of tumours of the anterior pituitary gland, liver, breast, lung and pancreas can be sharply reduced by altering the availability of food or reducing its nutritional quality by mixing it with a suitable inert ingredient. If fed in such a way, animals live longer and appear more healthy; their increased survival may also reflect a lower incidence of non-neoplastic degenerative conditions such as 'chronic progressive nephropathy'[15]. One approach has been to aim to hold rodents at 80% of the body weight of their free-feeding counterparts—ie at a nutritional level that does not harm their welfare and which, by greatly reducing the occurrence of spontaneous tumours, will materially enhance the statistical power of the test (see Chapter 6). The mechanism of these effects is obscure but reduced secretion of prolactin and perhaps other hormones released from the hypothalamic-pituitary axis is probably involved.

The most suitable nutritional regime, or the desirable body weight, have yet to be agreed because of the paucity of information. There is, therefore, no general agreement about the value of altering the dietary regime in carcinogenicity testing. The decision to do so must rest at present with the individual toxicologist. The main reasons for caution are that the consequences of dietary restriction even on the responses to known carcinogens, particularly in terms of their kinetics and metabolism, are insufficiently documented, and the logistics of such a procedure may prove difficult. The appropriate duration of experiments in such longer-lived animals is also not known.

If the test substance is formulated in the diet it is important to demonstrate that the compound and the process of preparation have not affected the normal balance and/or availability of essential constituents such as minerals, vitamins and fatty acids. This can be particularly difficult if a food component or inert ingredient is to be tested, when setting the maximum dose level may have to take nutritional balance into account. Where the substance has nutritional value it may be necessary to formulate the diet around the test chemical; a group of animals maintained on a suitable control diet would have to be included in the test design. If the substance is an amino-acid analogue, anti-vitamin or chelator of trace elements (and such properties may have bearing on the toxicity induced) then consideration should be given to the composition of diet on a case-by-case basis.

If the test material is a solid, dosing in the diet will require use of sufficiently fine particles to prevent their identification and rejection by the animals. Particulate materials in inhalation studies should be tested in their manufactured form but it is essential that the size distribution of the particles is documented with particular reference to the respirable range.

Test substance

The toxicologist needs to have an analytical specification for the material supplied (or a 'fingerprint' if it is a mixture), its principal impurities, and any solvent or suspending agent used to make up the dosing formulation. Stability must be checked at appropriate intervals, especially if a diluted preparation is administered or the substance is admixed with diet. Several different batches are likely to be required during a prolonged test, and the comparability for their composition and stability will need to be established.

Randomisation

When the animals for the experiment have been chosen and the experimental groups defined by the experimental design, then allocation of animals to their respective experimental group should be randomised. Both computer routines and published tables are available to generate pseudo-random numbers for this purpose. Randomisation procedures in transplacental testing are much more complicated. The aim of the randomised allocation of animals to experimental groups is to ensure that any bias in the allocation is eliminated.

Additional randomisation is sometimes performed in the location of the cages in which the animals are housed, to guard against bias generated by positional effect (eg intensity of lighting).

Stratification

The power of the study may sometimes be increased by stratification of the animals by weight or other variables that require balancing between the groups. Animals should be stratified by litter when using outbred animals to control for genetic variation.

Controls, dose groups and doses

In a carcinogenicity experiment, as in any very prolonged study, there is always an appreciable incidence of spontaneous neoplasms and other disorders which are not produced by the treatment—see Chapter 6. Their occurrence and other effects in the animals may (at least in part) be influenced by environmental factors such as husbandry, proximity to other animals and stress. Concurrent control groups are therefore essential to permit allowance to be made for these variable nuisance factors by providing an adequate basis for statistical comparisons. An acceptable practice is to set up two identical but distinct control groups, each comprising at least 50 males and 50 females, housed and treated in exactly the same way as the test animals except that they receive only the vehicle instead of the formulated test material. The reason for having two independent groups is that they will indicate the innate variability in the incidence of spontaneous lesions. The issue of concurrent and historical controls is considered in Chapter 6.

It is generally advisable to have three treatment groups (low, intermediate and high dose) so that the dose-response relationship of any effect can be examined. Each should contain at least 50 male and 50 female animals. The difficulties of choosing appropriate doses have already been mentioned. The general aim is to maximise exposure, tempered by toxicokinetic and toxicity considerations; but it is equally important, especially in oral tests on inactive substances or macro-ingredients of the diet, not to disturb the normal nutrition and health of animals by giving too large a dose or by making the diet unpalatable. Selection of the levels for the high, intermediate and low dose groups is usually done by setting the *high dose* at the maximum tolerated dose (MTD), the *low dose* at a small multiple of the likely human exposure, and the *intermediate dose* at their geometric mean (or at a level which will maximise the value of the data generated in the study eg a multiple of the human exposure for a drug)—see section on Toxicokinetics.

The maximum tolerated dose is selected first by reference to the toxicokinetics of the compound (or a marker component in a mixture); it should in general be as high as possible provided that other undesirable effects are not found, eg saturation of normal absorptive or metabolic pathways. Next, evidence about the toxic effects of that dose level over a period of several weeks to a few months should be reviewed. A dose producing continuing necrosis or other severe tissue damage needs to be avoided because it will jeopardise survival and compromise interpretation of the results. If the substance does not cause specific target organ toxicity or a gross functional effect, the high dose level is conventionally set as the dose likely to cause about 10% reduction in weight gain over the life span of the animals to be used; it is commonly chosen from the results of a preliminary 90-day toxicity test and multi-dose kinetic studies. A further constraint is that the top dose of a substance should not normally exceed 5% of the weight of the diet or drinking water, if administered in either of these vehicles, for reasons of nutritional status and welfare. If a substance is to be given in drinking water or diet it may be necessary to adjust the concentration at regular intervals to ensure a reasonably constant exposure throughout life.

The reasons for selecting particular doses should be clearly stated in the final report, including such relevant toxicokinetic data as blood levels, areas under the curve and metabolic patterns.

For many substances administered by inhalation, injection or topical application, the maximum dose may physically be limited by the process of administration. In such cases, the adequacy of systemic and local exposure should be compared in order to assess the need for separate studies by the topical and a systemic route.

In general, the use of *positive* controls and comparator substances is not necessary. Their occasional value lies in demonstrating that a particular study was able to reveal an unusual action, in investigating the mechanism of an effect, and (very rarely) in exploring interactions—either of the individual components in a complex mixture, or in pharmacological exploration of a mechanism.

Route of exposure

Animals should be treated by the likely route of exposure of man, at least as far as inhalation, oral and cutaneous exposure are concerned. For industrial chemicals it is usually better to employ oral rather than cutaneous dosing, unless there are special reasons to investigate local effects—for example, a compound where human exposure will be confined to the skin. Oral dosing will be simpler, better controlled and less stressful, and adequate systemic exposure is more likely to be obtained. In the uncommon instance of a need to test pharmaceuticals which will be administered parenterally, *per vaginam*, or *per rectum*, consideration should be given first to using an easier route (if supported by toxicokinetic and dynamic information), and then to a simpler but equivalent treatment site—for example intraperitoneal instead of intravenous injection.

Duration of exposure

The toxicologist has to address two separate questions here: when to begin and when to stop. It is conventional to begin dosing shortly after weaning (ie during early adult life) and to continue treatment unchanged for 2 years for the rat and 1.5 to 2 years for the mouse and hamster. These periods have been chosen on empirical grounds to balance the conflict between the wish to continue treatment for a long period to increase the likelihood of detecting an oncogenic effect, and the problems of ageing and intercurrent deaths from spontaneous tumours and non-neoplastic diseases which become increasingly common in old animals. Longer periods of dosing have been employed and, provided that the health of the animals is maintained, their value cannot be gainsaid. Briefer exposures are inadequate. A reasoned decision must, however, be made at the onset about the end-point of the test, irrespective of whether it is based on the number of months on treatment or on percentage survival in a specified group[5].

Exposure of the fetus by dosing the dam to weaning, and then continuing to administer the test substance to the weaned pups, has been employed from time

to time. It has not generally been found to be especially valuable except for hormonally active carcinogens such as oestrogens which may cause embryonic maldevelopment and dislocation of organ precursors. Variation in sensitivity during pregnancy and post-natal development makes it difficult to choose suitable doses of the test compound. This type of procedure carries additional problems, including uncertainty about exposure early in life, adequate group sizes and inbreeding in multigeneration studies. Statistical analysis of results is also difficult (see Chapter 6).

Frequency of dosing

Knowledge of the kinetics of the test compound may determine the appropriate frequency of treatment. *Oral treatment*, by incorporation in the diet or drinking water, should preferably be done every day. Gavage dosing may cause stress and practical difficulties and its use should be carefully considered. It is customary to limit *inhalation* exposure to 6 or sometimes 8 hours per day, often on 5 rather than 7 days per week. Skin painting or other *cutaneous* or *topical* treatments are normally undertaken twice weekly, unless local tissue reaction forces less frequent application. *Parenteral injection* is now an uncommon route but, if required, doses should be given daily.

Reversibility

Study of the reversibility of a tumorigenic effect is not part of a standard experiment, but it may provide valuable evidence about safety for substances that act by disturbing endocrine or other physiological feedback controls. If previous toxicity findings suggest such a mechanism, it may be helpful to include extra animals dosed for the full period or at least for long enough for benign tumours to develop and then to leave them untreated for some months to demonstrate complete or partial regression.

Observations in carcinogenicity tests:

Pathology

The basic purpose of carcinogenicity tests is to provide information about tumour incidence—assessed in terms of numbers, time of appearance and histological type—in groups of treated animals and in untreated controls. Appropriate laboratory and physiological observations may also be made to detect or exclude other biological actions and toxic effects of the substance, but their utility should always be assessed against the disturbance they may cause, and the difficulty of interpreting the highly variable results common in any population of ageing animals. Blood and/or urine samples may be taken at intervals throughout the study to confirm consistency of exposure and possibly to be used in tests to demonstrate whether organ toxicity has occurred. Use of satellite groups may be helpful for these purposes.

It is standard practice to record survival, weight gain, clinical signs and behaviour, and the development of palpable and visible masses.

Autopsies must be conducted according to a standard protocol which includes weighing of major viscera. The minimum set of tissues needed for microscopy is discussed in various published guidelines[1-8]. Paraffin wax-embedded sections of formal-saline fixed tissues remain the standard histological preparation but the use of additional or alternative fixatives should be anticipated—for example, Bouin's solution for the testes. Organs such as the brain and spinal cord, nasal cavities, lungs, bladder and intestinal tract need careful fixation, often with inflation of hollow viscera. Multiple slices should be examined to exclude the possibility of missing small or sparse lesions before the fixed tissues are sent for processing. The lymphoid system needs to be widely sampled by examining lymph nodes from different sites and gut and bronchus-associated lymphoid tissues, as well as routine blocks from the spleen and thymic region. The bone marrow should also be examined. If a substance is already known to affect a particular target organ it may be helpful to collect and store extra material for appropriate immunohistochemistry, electron microscopy and other investigations.

Histopathological examination should preferably be carried out on tissues from all groups of animals; but it is acceptable, in the first instance, only to examine all tissues from the high dose and control groups and of any macroscopically abnormal tissues in any other animals. Histopathological changes should always be assessed in conjunction with the clinical history, laboratory findings and the macroscopic appearances at autopsy, so that the various changes can be correlated. The diagnostic criteria and nomenclature used by the pathologist must be standardised and, in rare or debatable lesions, adequately justified.

To attain a valid comparison the same numbers of tissue samples and histological sections, all of the same size, should be examined from all animals.

Intercurrent deaths

The issue of intercurrent mortality is dealt with in Chapter 6. The age adjustment required for a proper comparison of tumour incidence between groups requires that a decision is made about the cause of death of each animal. If a tumour is fatal, the denominator is the number of animals alive without observable tumours; conversely if the tumours are incidental the appropriate denominator is the number of animals dying during a particular time interval. In order to reduce the chance that intercurrent death will result in autolysis (and for humane reasons), the majority of animals on a carcinogenicity study are killed when they show signs of illness rather than allowed to die.

The policy adopted as to when animals are killed (eg in regard to externally observable but non-life threatening tumours) can alter the power of the study, and this should be taken into account in the design and interpretation of the study.

Some incidental problems and practical requirements

Variations in husbandry, diet or environment of the animals may occur—for example, if there is a failure of environmental control in the animal house. The

event must be documented but it is unlikely to affect the outcome of the test unless the health and survival of the animals are materially affected. Three problems with potentially serious effects are:

i. *Inappropriately high dose levels*
This is usually the consequence of inadequate previous investigations into toxicokinetics and metabolism of the test compound and inadequate subacute toxicity testing. The only answer is to reduce the dose levels, perhaps after a brief period without treatment. The final results may, however, be jeopardised and, if there is a high associated mortality and morbidity, the test will have to be started again.

ii. *Intercurrent infections*
Despite every care infections (particularly viral) may occur. The risk is enhanced if the animals are severely stressed or immune-suppressed as a result of treatment. If there is overt disease the causal organism(s) should be identified if possible, as certain viral infections may influence the development of tumours at specific sites. If many animals die or need to be killed prematurely the experiment may have to be stopped. Chronic lung disease (*Mycoplasma* spp. and secondary bacterial infection) should not be an important problem nowadays in rats and mice.

iii. *Substandard pathological examination*
Several factors may be involved, including loss of animals from autopsy as a result of autolysis or cannibalism; poor fixation of tissues; inadequate sampling so that lesions which are small, sparse or have developed at unusual sites are missed; and failure to correlate gross and microscopic findings. These failings jeopardise the value of the experiment and may make it unsuitable for regulatory evaluation.

Additional important practical points

i. *Regulatory requirements*
In addition to the ethical and legal requirements concerning animals experiments, the test should conform to the principles of GLP[10].

ii. *Organisation*
Adequate numbers of trained staff at various levels are required to undertake husbandry, dosing and observation of all the animals in life and at autopsy; to perform the histopathological examinations; and to provide the necessary biostatistical expertise to advise on experimental design and to analyse the results.

iii. *Safety*
It is necessary to maintain a high standard of hygiene and appropriate practical procedures to prevent exposure of staff to harmful substances, including application of the COSHH regulations[16] and other safety guidelines[17–19], devising procedures for safe cleansing or disposal of unwanted compound and contaminated bedding and equipment[18]. Every unknown test substance should be regarded as hazardous and handled accordingly, until proven otherwise. The need for care has been well demonstrated by the

extent of unrealised dissemination of an inert test compound in an experienced laboratory[20].

iv. *Animals*
The species, strain, and supplier must be agreed in advance and the availability of sufficient animals confirmed, including any additional groups required for health checks, interim observations or early phase replacements. Their health status should be defined.

v. *Dosing materials*
An adequate supply of formulated material—the test compound, vehicle and excipients—must be available together with adequate methods for their analysis.

vi. *Record keeping*
The adequacy of collecting and recording all observations must be assured, irrespective of whether manual or automated systems are used.

Variations in the standard test system

Unusual substances

There are practical problems in investigating *volatile*, *corrosive* or highly *irritant* or *allergenic* materials in carcinogenicity tests.

The inhalation route is preferred for the controlled administration of volatile substances, but they can be administered by the oral or parenteral route, given appropriate technical precautions in formulating and dispensing the material. This may, however, present difficulties when extrapolating from the dosage to human exposure. Care and special procedures will be necessary to prevent contamination of other animals and the staff. Measurement of the true dose may well require parallel experiments in sealed systems. Corrosive and allergenic materials will require additional safety precautions for the staff who handle them and special consideration for the welfare of the animals used. Considerable dilution of a particular formulation may be needed to prevent excessive local damage at the site of application such that a valid result could not be obtained; alternatively, multiple small daily doses might be used which are too small individually to cause immediate injury. Highly toxic materials are also difficult to test because the tolerable dose will be low. The only practical approach is to explore the value of multiple, very small doses, chosen on the basis of previous toxicokinetic and toxicity data.

An unfamiliar vehicle or excipient may itself require testing, and even seemingly bland agents can have undesirable effects—for example, the ability of oral corn oil to enhance the development of tumours in the exocrine pancreas and at other sites in certain strains of rat.

Solid materials and devices

These materials for implantation in the body, usually in the form of prostheses, pose particularly difficult problems for the toxicologist. Plastic polymers, for example, will readily induce local sarcomas at the site of implantation in rodents; but the underlying mechanisms are unclear and a positive result in animals may not necessarily indicate a human hazard (Chapter 1). If such materials must be tested it is suggested that they should be implanted subcutaneously in the form of particles which are less likely to excite 'solid-state' carcinogenic processes. The mouse is preferable to the rat as it appears to be less prone to develop sarcomas as a consequence of the physical form of the implant.

It is not possible to give any specific guidance on the testing of solid implants, and the studies appropriate for a given device will need to be identified on a case-by-case basis.

The testing of essentially insoluble solid material for 'systemic' carcinogenicity (as opposed to local effects) presents many practical difficulties. The concept of a maximum tolerated dose, and the associated physical size of the implant, make any 'routine' approach impossible. The probable low 'leachability' of any constituents will require methods involving implantation or the testing of extracts, which may have low sensitivity to any individual constituent of concern. It is of crucial importance to consider the individual components of the device, and whether adequate data are available both on their mutagenic and carcinogenic potential, either from experimental studies or from long history of clinical use, so as to provide reassurance in this regard. For novel compounds, and those for which the existing data are inadequate, these components will need to be investigated for mutagenic potential and for carcinogenicity in animal bioassay(s).

There may be instances where the solid implant needs to be investigated in a way which mirrors the in-use situation, the objective being to define whether local tumours may occur (solid state tumorigenesis). However, such studies will need to be carefully adapted to suit the specific instance in question, and no general guidance can be given.

Products of recombinant DNA (rDNA) technology

These products, such as cellular growth factors, pose special problems. They are complex, high molecular weight substances, normally proteins, which are increasingly being introduced for industrial and pharmaceutical purposes. The reasons for testing them for carcinogenicity will be the same as for any other substance to which man may be exposed. There is no inherent property of the process of genetic modification of DNA (or of intact cells and organisms and their products) that would itself produce a tumorigenic risk requiring special evaluation.

When evaluating the possible carcinogenic risk of the final product of rDNA technology, it is important as in all other cases to consider its biological

properties and the pattern of human exposure. With regard to medicinal products, for example, the European Committee on Proprietary and Medicinal Products[21] recommends considering whether the substance can stimulate cell division, which is a necessary but far from complete mechanism in oncogenesis; whether it can produce sustained cell proliferation and under what circumstances; and whether its use by humans would be likely to permit any such actions to be expressed in cells capable of tumorigenesis, ie if it could act on a self-renewing population which was not committed to terminal differentiation. The decision to consider oncogenicity testing would depend then on unphysiological (pharmacological) exposure to a substance capable at least of producing sustained proliferation of uncommitted cells. However, (as discussed in Chapter 1), potentiation of mitogenic activity on its own cannot be regarded as a tumorigenic effect although it may 'promote' cells already 'initiated' by other means. That process is of uncertain relevance to man, although it might be worth exploring under appropriate circumstances.

The nature of the materials creates a further, serious problem in planning an oncogenicity test. It would be essential to use a species in which the appropriate pharmacological action was manifested, and many of the products show a narrow range of specificity that excludes laboratory rodents. They are also often antigenic in foreign species, and the almost inevitable development of an immune response in test animals is likely to make it pointless to continue the study—either because of the risk of immune damage, or because the activity of the substance will be greatly altered or even neutralised.

For all these reasons, a conventional type of carcinogenicity study will rarely be feasible or useful. In the future, it may be worthwhile exploring effects in transgenic animals strongly expressing the factor (and any appropriate receptor), or even to employ a locally expressed oncogene—at a site appropriate to topical application of the substance—but at the moment such approaches belong to exploratory research and not to hazard assessment.

Unusual routes of administration

Most substances are tested orally, by inhalation or by topical cutaneous application. It is however, sometimes necessary to consider preparations intended solely for administration *per rectum* (*pr*), *per vaginam* (*pv*) or by the ocular, nasal or aural routes. It would be arduous and stressful to dose animals *pv* or *pr* in a lifetime study, and every attempt should be made to justify use of the oral or cutaneous routes instead by comparison of the kinetics and local tissue availability. Similarly, ocular and aural administration should usually be covered for carcinogenicity testing purposes by an oral test. Nasal exposure is more problematical because of the diversity and discrete reactivity of the tissues in the nasal cavity. It would probably be best to consider a study by the nasal route, using either instillation of droplets or exposure to an appropriately sized aerosol, although either would inevitably entail extensive gastro-intestinal dosing as well. Studies are sometimes done involving intratracheal, intrapleural or intraperitoneal injection of materials. They have been primarily employed in investigating metallic and other dusts and mineral fibres, and they are not

recommended for the routine assessment of these or other types of materials. Their greatest value has probably been in accelerated tests and mechanistic investigations rather than in providing data for practical risk assessment.

Susceptible strains of animals and accelerated tests

The inherent propensity of Strain A mice spontaneously to develop lung tumours can be greatly enhanced by relatively short-term treatment with carcinogens[22]. The procedure has been proposed as a speedy general method for indicating carcinogenic potential, but it does not appear to be sufficiently discriminative for general use.

The Sencar strain of mouse has been specially selected for its sensitivity to the cutaneous application of the carcinogenic polycyclic aromatic hydrocarbons which will induce skin tumours in this strain in 2–3 months. Knowledge of the responsiveness of Sencar mice to other classes of chemicals or to a range of vehicles is too limited to recommend their use in carcinogenicity testing for regulatory purposes.

Assays for two-stage carcinogenesis: initiation and promotion

Assays for tumour initiating and promoting activity have been available for many years, using mouse skin as a target tissue. More recently, two-stage models have been devised for other rodent organs such as the liver, bladder, kidney, lung and thyroid[23]. These systems were originally devised to analyse various stages of tumour development in an experimental context. The theoretical basis for two-stage models of carcinogenesis is debatable (see Chapter 1) and experience of their use in a regulatory context remains negligible. At least some aspects of tumour promotion can now be analysed in tissue culture[24,25] and it is feasible that short-term screening tests for non-genotoxic carcinogens will eventually be available for regulatory purposes (see also Chapter 4).

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6. Interpretation of carcinogenicity studies

A wide range of information is usually available to help the toxicologist to assess the carcinogenicity of a substance, relating to its chemical structure or composition, physical properties, toxicokinetics, effects in acute and sub-acute toxicity studies, and mutagenic activity in a series of *in vitro* and *in vivo* assays. Some human data may be available from epidemiological investigations, but in their absence the major source of information is provided by long-term carcinogenicity tests. These studies may be designed simply to assess in a qualitative fashion whether the substance has carcinogenic properties, or to provide information on mechanisms of action and other data suitable for a quantitative assessment of hazard. They represent a substantial investment in time and skilled human resources and it is essential to extract as much relevant information as possible.

Conduct of the study

A single long-term carcinogenicity bioassay, particularly if it has included an assessment of the chronic toxicity of the test substance, can yield over 500,000 data points. Such studies should ideally be conducted according to the principles of Good Laboratory Practice (GLP) which aim to ensure that the completed report reflects accurately and comprehensively the conduct of the investigations. (It should, however, be noted that GLP does not automatically quarantee high scientific quality). Reports prepared in this way are now required for regulatory submissions.

In addition to GLP-based reports, published work from the open literature may also be available for evaluation. Such papers will have been submitted to previous peer review, but the studies on which they are based may not have been conducted according to GLP principles. It is therefore essential that evidence from publications of this kind should be carefully scrutinised and, wherever possible, the principles of data verification and inclusion of key points in reporting (which are the hallmark of GLP) should be applied. Examples include details of experimental design in relation to the *animals* (sources, strain, health status, group size and randomisation); the *test substance* (purity, stability, evidence of adequate dosage and exposure of target tissues); the *experimental environment* (temperature and humidity, stocking density); and the *general conduct* of the study (comprehensiveness of pathology, and the relationship of histopathology to findings at autopsy). Tabulated data from any source need to be scrutinised for numerical inconsistencies such as 'missing' animals or 'missing' tumours. Authors of these publications and editors of journals should

be encouraged to ensure that sufficient details are included so that reviewers can satisfy themselves about these key elements.

Exclusion of confounding factors

Several potential confounding factors in long-term carcinogenicity tests were identified in Chapter 5. Most of the factors which may affect the assessment of the relationship between dose and endpoint (ie tumour development) should have been anticipated when the individual studies were being planned; but if unavoidable, they should be included in the protocol where their impact can (if necessary) be gauged by appropriate statistical analysis. Confounding factors may have an effect not so much on assessments of cause and effect in the experiment under consideration, but on the eventual interpretation of the study for the purpose of hazard assessment. In many cases they may be difficult to identify and will require expert interpretation in the final hazard assessment.

The confounding influence of certain aspects of experimental design may not be apparent until the histopathological examinations are completed. The selection of the top dose in carcinogenicity tests is difficult; unexpected tissue damage may be sustained which, both in terms of its occurrence and its subsequent evaluation, could not have been predicted from previous subacute toxicity studies. Equally, evidence of sustained tissue damage from such an occurrence may influence the interpretation of the results from the point of view of hazard assessment. In some circumstances perturbation of physiological function must be taken into account—for example, in inhalation studies where excessive numbers of respirable particles may progressively impair the normal clearance of particles from the lung by the muco-ciliary mechanism. The incidence of pulmonary tumours may be increased in such circumstances but their significance for humans exposed to much lower numbers of particles may be difficult to interpret. It is important to consider the effect of excessive particulate overload and how this relates to the less extreme conditions experienced by humans, where the clearance mechanisms may not be seriously compromised.

Irrespective of the mechanisms involved and the target organs affected, treatment-related tissue damage which is gross and sustained may accelerate the later stages of tumour development; tumour incidence is thereby increased by a process which is not necessarily relevant to man or other species exposed to lower doses in less contrived circumstances. Examples include the increased incidence of skin tumours following chronic local irritation and bladder tumours associated with urolithiasis in rats caused in turn by excessively high doses of lithogenic chemicals.

Environmental conditions, defined broadly, can profoundly affect the incidence of neoplasms and so confound the interpretation of results. Differences in food intake, which may be associated with unpalatability, can influence the tumour incidence[1]. Therefore it may be difficult to separate the direct effect of the chemical from the confounding influence of the reduction in dietary intake. Good experimental design should aim to avoid these effects and monitor food

consumption so that the confounding effect of any reduced dietary intake can be assessed.

Many test substances are not water soluble and are dissolved in a vehicle when incorporated in the diet or administered by gavage. An increased incidence of certain tumour types may be associated with the vehicle—for example, the pancreatic acinar cell adenomas found in control male F344 rats given corn oil[2]—an evaluation of appropriate controls is essential. The vehicle and route of administration may exert major effects on the toxicokinetics and metabolism of the substance. This will require substantial knowledge of the variation of target tissue concentration of the compound and its metabolites with time in both the experimental animal and (if possible) in man, for an adequate assessment of hazard.

Identification of critical biological findings

Once confounding effects which may compromise the interpretation of the data have been identified, analysis of the toxicological significance of the results can begin. The prime requirement is that any apparent dose-related increase in the incidence of each category of neoplasm is rigorously evaluated. The use of a 5% significance level in a two-tailed test as the sole criterion for judgement is often inappropriate, and Haseman[3] reports that the U.S. National Toxicology Program's Peer Review Panel decisions approximate to a 1% significance level. This is probably because the statistical analysis of a standard carcinogenicity study involves several hundred pair-wise comparisons which will inevitably result in a number of differences due to chance which are significant at the 5% level. For these reasons, it is desirable to have the results of the statistical analysis reported as p-values or in terms of a number of different cut-off points ($p < 0.05$, $p < 0.01$ and $p < 0.001$). The presence of a dose response relationship aids considerably in considering the significance of any increase in tumour incidence. The distinction between a chance observation and a dose relationship when the increase occurs only in the top dose may be problematical. Similar responses in the other sex or in other species may aid interpretation.

Some of the neoplasms that develop are peculiar to rats and mice, such as granular cell tumours of the brain and neoplasms arising in the Harderian gland, Zymbal's gland and the forestomach. Specific types of compound may induce new or previously unfamiliar types of neoplasms such as mesovarian leiomyomas, gastric carcinoids and olfactory neuroblastomas. A major interpretative difficulty is posed by studies in which the target site in the test groups is also the site for appreciable numbers of similar 'spontaneous' tumours in untreated controls; some examples are listed in Table 6.1. Attempts are sometimes made to distinguish between such tumours in test and control groups in terms of their numbers per site, their relative time of onset, and the proportion of benign and malignant neoplasms; all are unreliable. The incidence of the common spontaneous tumours is notoriously variable and it may be very difficult to assess whether there is a compound-related effect. The presence of a dose-relationship and additional data from historical controls may be useful here in providing biological information to supplement the

Table 6.1: *Incidence of some common naturally-occurring tumours in untreated F344 rats and B6C3F$_1$ mice*

Tumour site/type	Sex	% Tumours	
		range	overall mean
F344 rats *Data from 72 control groups from 6 laboratories*			
Lung	M	2.2– 5.8	3.7
	F	0.7– 4.6	2.2
Lymphoma/leukaemia	M	9.1–23.6	16.9
	F	7.5–15.4	11.0
Liver	M	0.7– 3.4	2.0
	F	0.5– 2.9	2.0
Pituitary	M	7.5–31.2	20.3
	F	31.0–58.6	40.0
Thyroid (C-cell)	M	3.6– 9.0	7.1
	F	4.7– 7.0	5.8
Adrenal medulla	M	4.0–16.0	10.7
	F	1.0– 5.6	3.2
Pancreatic islets	M	2.8– 9.0	4.9
	F	0.3– 1.9	1.1
Breast (fibroadenoma)	F	6.4–24.2	15.5
B6C3F$_1$ mice *Data from 54 control groups from 7 laboratories*			
Lung	M	10.6–21.9	17.0
	F	3.6– 7.1	6.0
Lymphoma/leukaemia	M	7.2–12.2	11.2
	F	17.0–30.4	24.4
Liver	M	25.0–40.1	32.1
	F	4.6– 9.7	6.2

All groups contained 20–50 animals per sex; killed at 110–116 weeks
Data compiled from Tarone *et al*[20]

statistics and aid interpretation[4]. Data from historical series can, however, be difficult to assess even with adequate numbers of animals accrued over an acceptable time-span. The incidence of certain spontaneous tumours varies markedly with conditions of animal husbandry[1,5] and criteria for pathological diagnosis may shift—a situation illustrated by the changing approaches to defining hyperplastic lesions and tumours in the liver of the rat[6,7].

Non-neoplastic pathology and the patterns of neoplastic response also provide valuable information. For example, evidence of early organ toxicity, followed by an increased incidence of tumours at the same site, may reflect either altered patterns of metabolism or mechanisms of action; both require cautious assessment in considering their potential significance in humans.

The distinction between hyperplasia, benign tumours and malignant tumours is central to the interpretation of carcinogenicity studies. Some chemicals produce a neoplastic response with a clear progression from hyperplasia to benign tumours to malignant tumours. Thus an analysis of malignant neoplasms or the combination of benign and malignant neoplasms is appropriate. In other instances where there is no evidence for progression and where the major increase is seen in benign tumours, separate analyses of the two tumour categories may be necessary. The differential diagnosis of hyperplasia and benign tumours or of benign tumours and malignant neoplasms may be arbitrary. For example, the distinction between hyperplasia of Leydig cells and Leydig cell adenomas may have to be based on arbitrary categories of lesion size. Another example is provided by thyroid neoplasms where certain chemically-induced tumours which are classified as locally malignant by conventional histological criteria may regress if treatment is stopped. Interpretation in this particular instance will rely more heavily on biological and toxicological judgement. In general terms, a decision to combine or separate the three categories of hyperplasia, benign tumour and malignant tumour for the purposes of statistical analysis should be made on available data and on a case-by-case basis. The need for high quality histopathological material, standardised nomenclature and classification will be self-evident.

A *negative* study cannot automatically be interpreted as proof of a negative effect and attention to the design and performance of such bioassays is critical. A negative experiment, adequately designed and reported, should give reasonable confidence that the compound under investigation is not a carcinogen within the conditions of that particular bioassay.

Similar results from two test species provide greater confidence in extrapolation of data to human contexts. Differences in response between species, on the other hand, can lead to valuable mechanistic information which will assist in the assessment of likely human hazard.

Mechanisms of action

Information on the probable mode of action of a test compound should always be sought. It will be derived from standard mutagenicity and carcinogenicity tests and, in some instances, from a consideration of the substance's chemical and physical properties and from toxicokinetic studies. A distinction between genotoxic and non-genotoxic mechanisms is critical. Typical genotoxic carcinogens have an electrophilic structure or will generate electrophilic metabolites; they will induce mutations or clastogenic effects in short-term tests; and they will show a clear carcinogenic response in animal tests, usually in two species and often showing a predilection for certain sites. Some target organs in rodents appear to be particularly susceptible to tumour induction by genotoxic compounds, in contrast to others (such as the mouse liver) which are equally susceptible to both genotoxic and non-genotoxic agents[8]. Separation of these two classes of carcinogen is, however, sometimes blurred. Some substances may generate genotoxic moieties indirectly, and others exert a combination of genotoxic and non-genotoxic effects; examples are cited in Chapters 1 and 3.

Non-genotoxic carcinogens must, by definition, have given securely-based negative results in an adequate range of short-term tests which have been satisfactorily performed and interpreted (see Chapters 1 and 4). Positive evidence of a mode of action is often tenuous, but should be provided wherever possible. The most convincing examples are associated with alterations in normal physiological homeostasis. They include compounds which provoke chronic over-stimulation of the thyroid gland by thyroid stimulating hormone[9]; substances such as unleaded gasoline that bind to $\alpha_{2\mu}$ globulin which accumulates in an altered form in the kidney tubules and induces renal epithelial tumours specifically in male rats[10]; and hypolipidaemic agents which induce hepatocellular tumours in rats and mice as a consequence of sustained proliferation of peroxisomes[11].

The importance of selective long-term tissue damage and regeneration has already been noted although the underlying biochemical, cellular and subcellular changes which contribute to tumour development in such circumstances are obscure (see Chapter 1). Examples in rodents include chronic chemical or physical damage to local tissues as a result of repeated parenteral injections[12,13] or from gavage[14]; the formation of calculi in the urinary tract[15]; and accumulation of functionally impaired macrophages in the lung[16]. Tumours developing in animals under these circumstances may not necessarily indicate a risk for humans, evaluation on a case-by-case basis is needed.

Any conclusions drawn as to the likely mechanisms of action of a test compound must take full account of the experimental protocol with respect to the dose, route of administration, toxicokinetics, metabolism and other biological effects. Deductions about likely mechanisms based on results obtained with very high doses of a compound, or given by an unusual route, should be made with caution. The advantages of at least a partial knowledge of modes of action of a given compound are, however, considerable. Some mechanisms are unlikely to operate in any foreseeable human context and, for non-genotoxic processes, threshold levels can sometimes be derived in the test species (see Chapter 7).

Biostatistical considerations

Two major publications have appeared from the IARC[17,18] in the past 10 years which deal with the analysis of results from carcinogenicity tests. The latter, in particular, provides a comprehensive review of the main problems to be addressed and gives a detailed account of the available methods.

The main aim of biostatistical evaluation is to determine whether exposure to the test substance is associated with an increase in tumour development. The most common carcinogenic response is an increase in the age-specific rate of tumour occurrence over some portion of the life-span of the animal which, in the absence of differential mortality from other causes, leads to an increase in the lifetime probability of developing a tumour. Tests designed to detect changes in the time of occurrence (eg tumour acceleration) without a change in the overall rate of occurrence should be employed when there is a clear prior

hypothesis that acceleration (rather than augmentation) of tumour occurrence is the expected response.

The standard experimental design for long-term bioassays comprises one control and more than one treated group, 3 being the usual recommendation (see Chapter 5). The most powerful appropriate test for detecting a dose-related increase in the age-specific rate of cancer occurrence is a trend test—see IARC[17,18] for an extended discussion of the appropriate methods. For experiments with only one treated group and a control group—a design which would generally be regarded as inadequate—methods for comparison of 2 groups analogous to those for the previous design are appropriate. Only in exceptional circumstances, requiring justification in terms of a clear prior hypothesis, would tests be appropriate which are designed to detect a different form of change in cancer frequency—examples include a threshold effect or dose saturation. The following aspects need strict attention in the analysis:

i. *Intercurrent mortality.* It is common in carcinogenicity bioassays for animals at the highest dose levels to exhibit different mortality patterns which are unrelated to cancer induction. Toxicity may cause increased mortality rates, although it is recommended that the highest dose used should elicit slight toxicity and no substantial reduction in longevity due to effects other than tumours. At doses with slight toxicity it is also common to observe reduced mortality rates, perhaps as a result of reduced dietary intake. In both situations it is essential to employ methods which allow for the fact that the denominators appropriate for age-specific comparisons of tumour rates will differ across the experimental groups, even if the groups were of equal size when the experiment was set up. A simple approach (reference 18, p76) is to subtract from the total number in each group, the number dying before the first tumour (of a type of interest) is observed in any of the groups being compared. This leads to different denominators being used for each tumour type. A fuller and superior method is full age adjustment[18].

ii. *Context of observation.* The context in which tumours are encountered is of prime importance. Biostatisticians tend to classify neoplasms into 2 groups: fatal tumours which are directly responsible for the death of an animal and incidental tumours which are not, in themselves, lethal. For fatal tumours the denominator appropriate for each observed tumour is the number of animals alive without an observable cancer the instant before the tumour is observed. Intercurrent mortality can be handled by the straightforward methods of survival analysis. For incidental tumours, the appropriate denominator is the number of animals submitted to autopsy at a similar time. Methods for fatal and incidental tumour analyses are fully described in both IARC publications[17,18]. In general, observed incidence of an individual tumour may include both fatal and incidental contexts: analyses should be carried out separately for each context of observation and the two analyses combined for the purpose of interpretation[17,18]. In the absence of information regarding the context of observation, analyses can be carried out assuming that all tumours were fatal, and then repeating it with the assumption that all tumours were incidental. The results of these analyses provide a bound for the complete analysis, assuming that the lethality of the

tumour does not vary with time. An alternative approach when the context is not available is described by Lagakos and Louis[19].

iii. *Multiplicity of tumour types.* Most chemical carcinogens induce tumours in one or (at most) a small number of target organs. Analyses based on all tumour types combined may not reveal specific effects and this approach is not advised unless the exposure is thought to have a general, systemic effect—related, for example, to changes in total caloric intake from the diet. The recommended procedure is to analyse each tumour type separately.

iv. *Multiple tumours in a single organ.* The main methods developed for the analysis of animal carcinogenicity experiments concentrate on the numbers of animals bearing a particular type of tumour, rather than the total number of such tumours in each experimental group. The latter number would count each tumour, when multiple tumours of a particular type occur in the same animal. The reasons for counting tumour-bearing animals rather than tumours *per se* are that multiple tumours within an animal are not independent events and that the animal is the essential experimental unit, randomised between the dose groups.

Methods have been developed for analysing the number of tumours per animal[18] for specific purposes in special experimental systems, but their use is not advised in the analysis of routine bioassays.

v. *Use of spontaneous tumour rates.* The examination of spontaneous tumour rates obtained from previous and, when available, subsequent experiments can be useful in assessing the possible carcinogenicity of a test compound if the analysis relative to the concurrent controls has led to equivocal evidence of carcinogenicity. This applies particularly in the case of tumours which rarely occur spontaneously; a small incidence in a treated group may not achieve statistical significance when compared to the concurrent control group, but nevertheless needs to be considered as an important (if rare) event, given the historical experience with similar untreated animals. Care must be taken in any assessment using background control data to ensure that the control rates come from experiments which are similar to the current bioassay in factors known to affect the magnitude of spontaneous tumour rates; also that no time trend in control tumour incidence is apparent in the studies selected. For rare tumours, underlying trends may not be easy to detect and the most appropriate and important comparisons must be those relative to the concurrent control group. Formal methods for incorporating spontaneous tumour rates into the analysis have been developed[18].

vi. *Transplacental administration.* The fetus may be particularly susceptible to some chemical carcinogens, and some carcinogenicity studies commence with administration before and during pregnancy. Analysis of the results from such studies is difficult because, for transplacental effects, the litter is the statistically independent unit in the analysis. One method of avoiding this problem is to select only 1 male and 1 female from each litter for continuation in the lifetime exposure part of the study; in this case each animal in the F_1 generation can be used in the statistical analysis as an independent observation.

Further aspects of statistical analysis

This section has concentrated on statistical methods for determining whether a carcinogenic effect has been induced in a bioassay. If, however, the experimental data are to be fully exploited, there are several other topics where biostatistical analyses may be highly informative. These include:

- Modelling the dose-response relationship (see Chapter 7).
- Changes in age-specific tumour rates as a function of age, and as a function of changing dose rates with time if exposure is not constant.
- The effect of withdrawing the exposure on subsequent rates of tumour occurrence is of particular interest.
- Combined effect of more than one agent.
- Analysis of auxiliary data such as body weight and patterns of growth of the animals in relation to dose.

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7. Risk assessment

Introduction

All activities in life carry some risk of harm to health but, for many of these activities, implicit dangers are accepted because there are some implicit or explicit benefits to the individual or to society in general. For certain activities there may be a risk to individuals or to population groups of developing cancer. Individuals or society may view these risks as more or less acceptable depending on their magnitude, whether they are imposed or voluntary, and on the benefits accrued from the activities which caused them. Nevertheless, it is important for society that expert analysis of carcinogenic risks is undertaken so that appropriate priority can be given to measures aimed at controlling or reducing the risk. Thus the assessment of potential carcinogenic risks posed by new chemicals (or by proposed new uses of familiar compounds) is an essential part of the work undertaken by committees which advise regulatory authorities and government departments. It is therefore appropriate that general aspects of this topic are discussed in these guidelines.

The *acute* toxic effects of a novel chemical can be predicted with reasonable accuracy from increasingly more sophisticated and sensitive toxicological investigations, because the dose used is directly comparable to the human exposure[1]. It is the significance to human health of repeated exposure to low doses of mutagenic and/or carcinogenic compounds that have been tested at much higher experimental doses that remains difficult to evaluate, particularly in precise quantitative terms. The difficulties are compounded by a frequent failure to draw a distinction between hazard and risk[2,3]. *Hazard* in the present context describes the intrinsic capacity of a chemical to cause an adverse effect on human health; *risk* is the probability of that adverse health effect occurring. The level of risk will depend on particular circumstances, determined by the nature and degree of exposure to the chemical in question. *Safety* is the converse of risk and, in common usage, refers to a situation of minimal risk. The assessment of risk requires, in the first instance, access to well-founded information on hazard, derived from human studies (whenever available) and full laboratory data. In addition, a clear appreciation of the likely circumstances of human exposure through which putative risks may be incurred is needed.

There is a tendency within society to exaggerate certain risks and virtually ignore others, even where the scientific estimations of these risks are of the same magnitude. The aggregation of many low probability events makes up a large part of the total risks which individuals are most likely to encounter, but inordinate weight is often placed on risks which may have low probability but high public perception. The public perception of the relevance of a risk of

cancer may differ markedly from the reality of that risk as determined from all the available scientific evidence. Cigarette smoking is an obvious example of one of the greatest avoidable risks to public health. A young adult male smoker has on average a 1 in 4 chance of dying from all causes prematurely as a result of his continued smoking. Contrast this figure with the risk of a member of the general public in the UK dying as a result of the permitted radiation emissions from a nuclear power station, which is roughly the same as being killed by a bolt of lightning (both at around 1 in 10,000,000). There are other examples of the public's perception of risk where the facts have either been misinterpreted or the statements about risk lack any scientific foundation.

Hazard and risk assessment

The sequential analysis of hazard and risk for a given chemical falls into four main stages which may be summarised as follows:

i. **hazard identification**	by review of toxicity data, the results of toxicity testing, and any knowledge of effects on human health.
ii. **hazard evaluation**	by determination of factors including the dose-response relationship, potency, species variation in susceptibility, mechanism of toxicity.
iii. **exposure evaluation**	by estimation (or modelling) of probable human exposure, routes of entry and levels of potential exposure.
iv. **risk estimation**	by combination of the animal and/or human toxicity data, with or without mathematical modelling, and an evaluation of any human exposure so that an estimate can be made of the likelihood (or magnitude) of any human health effects which may occur.

Identification and evaluation of hazard is based on human and experimental data, the nature, advantages and limitations of which have been discussed in preceding chapters. Information on direct human exposure is rarely available, and it may be necessary to estimate the risk value for a range of hypothetical human exposures or to determine the degree of exposure that would be associated with an acceptable level of risk. It is, however, always preferable to use sound human data for quantitative risk assessment, for example from epidemiological studies, even when information on exposure is incomplete.

Risk estimation

The probability that an adverse health effect will be produced by exposure to a hazardous chemical is determined by consideration of the toxicity profile of the chemical—particularly the nature of the toxic effect, its characteristics (in terms

of species variation and mechanism of toxicity) and the dose-response relationship. These data are necessarily assessed by a process of integration and the application of sound scientific judgement. If they are found to be of poor quality then no precise quantification of associated risks can be made. The concept of 'toxicity' is a broad one, given that all chemicals are toxic at some dose. The prime purpose of toxicity testing is to establish the conditions under which the chemical may produce an adverse effect such as cancer. The intrinsic toxicity of a chemical is not the only factor, but rather the likelihood that a given level of exposure to the chemical will be sufficient for this intrinsic toxicity to become apparent.

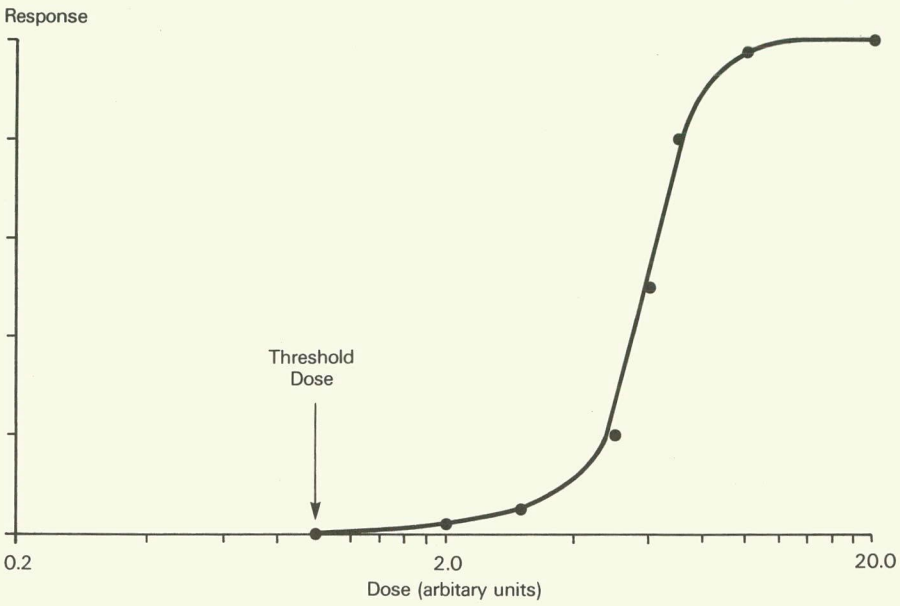
In certain circumstances, mathematical equations may be fitted to the data as an attempt to produce numerical estimates of risk from human exposure[4,5,6]. Many mathematical 'models' have been developed for use in assessing carcinogenic risk but they are only loosely compatible with our current (limited) understanding of mechanisms of chemical carcinogenesis and they have not been comprehensively validated. The models that are applied to carcinogenicity data attempt to define the probability range for the risk of developing a cancer at low dose by using statistical theory to extrapolate below the available experimental data points. The curves derived from the models are fitted to the available data points and then extrapolated to low doses with appropriate confidence bands. All of them attempt to define the dose-response relationship on the basis of a particular extrapolation of the experimental data beyond the lowest data point. These models are further discussed under 'Non-threshold toxicities'.

The likely human effects of exposure to carcinogens identified in long-term animal carcinogenicity studies are difficult to determine because of the extensive extrapolations of the data that have to be made. These include extrapolations *within* the test species from the high experimental doses used in the bioassay down to low doses in the range predicted for human exposure, and then extrapolations *across* species from rodents to humans. Furthermore, the results from animal tests are usually based on a single route of exposure which, although carefully chosen, may not adequately simulate the variety of exposure patterns that can reasonably be expected in humans. Species differences in kinetics and biotransformation also need to be considered.

Threshold and non-threshold toxicities

The way in which the safety of chemicals is assessed may vary with their mechanisms of toxicity which in turn affects the relationship between dose and toxic response. For most mechanisms it is accepted that there is a threshold dose, below which no toxicity is observed (see Figure 7.1). Some non-genotoxic carcinogens induce tumours as a secondary event following from a toxicological effect which has a threshold. It follows that, for these substances, there is no carcinogenic hazard at dose levels which do not produce the primary toxicological event ie at doses below the threshold.

Figure 7.1: Determination of threshold dose for toxic effect



It is prudent to assume that genotoxic carcinogens have the potential to damage DNA at any level of exposure and that such damage may lead to tumour development. Thus for genotoxic carcinogens it is assumed that there is no discernible threshold and that any level of exposure carries a carcinogenic risk.

Various non-threshold mathematical models have been developed to estimate the risk associated with exposures to genotoxic carcinogens. The Committee considers these models to be inadequately validated and does not use them for routine assessments at the present time.

For carcinogens which do not appear to be genotoxic but for which no mechanism of action has been established, the prudent approach of assuming no threshold for the carcinogenic effect is adopted.

Threshold toxicities

Hazard evaluation in most areas of toxicology is based on the no observable effect level (NOEL) in animal studies divided by an arbitrary *safety factor* to give an estimated safe dose in humans eg the derivation of an acceptable daily intake (ADI)[7]. The safety factor reflects the uncertainties of extrapolating findings in animals to humans and interindividual variation, and takes into account the quality of the toxicity data as well as the nature of the toxic effect. Its numerical value needs to be considered on a case-by-case basis, but as a general example a value of 100 is frequently used when based on good animal data. The value may be increased if the toxic effects of the compound give rise to particular concern or the quality of the animal studies is poor; it may be decreased if there are sound human data. Such an approach may be used for

non-genotoxic carcinogens provided that the underlying mechanisms are adequately understood. The safety factor approach, unlike the methods used for non-threshold effects considered later, does not generate a range of estimates (and their confidence limits) of risk but rather a single estimate of a dose (exposure) which is considered to be below the threshold dose likely to result in measurable risk; all exposures below this level are assumed to produce no increased risk. A dose above this level is assumed to present some risk, but the safety factor approach gives no estimate of the size of that risk for any particular dose.

Non-threshold toxicities: Genotoxic carcinogens

From what is known about the mechanism of action of genotoxic carcinogens, it is currently accepted that no threshold exists; risk estimates must therefore rely on extrapolation from high experimental doses to low exposure doses. One method of achieving this is by fitting one or more mathematical models to the dose response curve for the bioassay (at the relatively high doses necessary in experimental studies) and using the mathematical model to predict the response at very low doses. Many models are available which fall into 2 general classes:

(i) 'stochastic' or 'mechanistic' which are very roughly compatible with a broad range of experimental observations in carcinogenesis, and

(ii) 'tolerance' or 'empirical' which are based solely on curve fitting methods.

Calculation of low dose risk by a particular model does not reflect an understanding of the mechanism of action of the chemical concerned. All of the models reduce to a set of equations which define a family of curves describing dose-dependence and as such it is not possible to select a particular model as the 'best' on the basis of biological relevance. They will frequently describe the observed dose-response data equally well, but the predicted risks at doses below the experimental range may differ considerably, thus presenting problems about the relevance of the predictions. Table 7.1 summarises the categories of mathematical models.

Table 7.1: *Categories of Models for Carcinogen Risk Assessment*

Category	Model
Stochastic[a] (or mechanistic)	One hit Multihit Multistage Linearised Multistage Hartley-Sielken[c] Two stage model (Moolgavkar-Venzon-Knudson)
Tolerance Distribution[b] (or empirical)	Weibull Logit Probit (Mantel-Bryan)

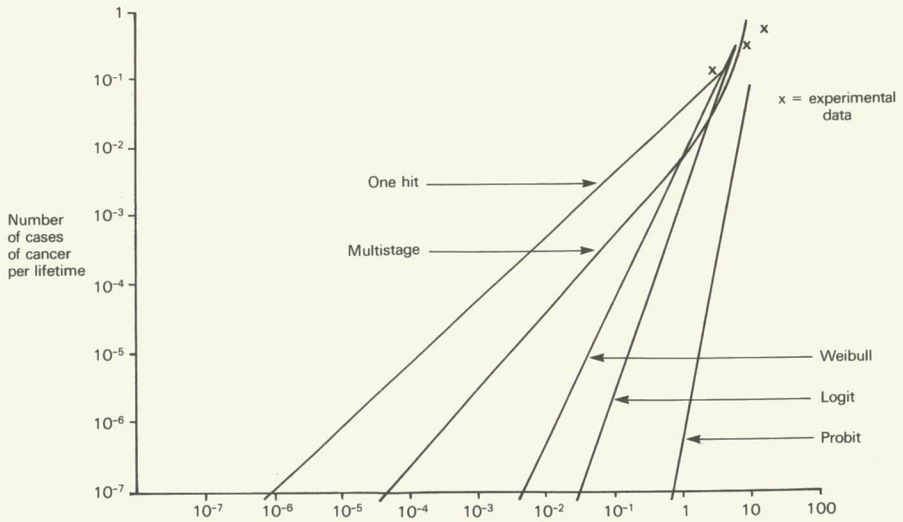
a. suitable for dose-response or time to tumour data
b. suitable for dose-response data
c. a model dealing with only time to tumour data

The stochastic models are based on the concept that a toxic response is the result of a random occurrence of one or more fundamental biological events. In the multistage model, it is assumed that the events take place sequentially and that there are one or more distinct events. Tolerance distribution models assume that each animal (or member of a population) has its own threshold and will not develop a tumour below this level of exposure. This threshold level varies from individual to individual according to a tolerance distribution. Time-to-tumour models describe the relationship between dose and tumour latency while the two stage model proposes that initiated cells arise from normal cells following a single mutation and then undergo clonal expansion (and probably further mutation to form a malignant cell). Some of these models are already used by some toxicologists and regulatory authorities; the two stage model requires much detailed information on cell kinetics which is rarely available and hence it has not been used extensively.

The differences in the models are apparent when estimating risk at low (extrapolated) doses, but estimates of low risks are difficult if not impossible to verify. In practice, because group sizes are limited to around 50 animals of each sex and spontaneous cancer incidence at some sites may be high, treatment-related increases in cancer incidence of about 10% in animal bioassays may be indistinguishable from the spontaneous tumour rate in that species or strain. To overcome the variations between the models a simplified method has been suggested—a model-free approach[8]—in which the risk is estimated by linear interpolation from one of the observed data points.

The models are generally linear at low doses. The variability at low doses in the fitting of a number of models is shown in Figure 7.2. This variability at low

Figure 7.2: Risk versus Dose



(redrawn from reference 9)

doses between the models used indicates that the risks described will vary markedly with the model chosen.

Some regulatory authorities (particularly in the US) use a linearised multistage model in which risk is assumed to be linear (directly proportionate to dose) at low doses. Figure 7.2 also indicates the 'conservatism' in using the multistage model—ie the tendency to provide high estimates of risk. The risk derived from these models may be calculated and expressed as the best estimate of fit (maximum likelihood estimate) or in terms of the 95% Upper Confidence Limit. The latter will indicate an upper boundary on the estimate of risk and has the characteristic that relatively small changes in estimated risk occur with alterations in the experimental dose-response information.

Although it is assumed that any exposure will carry some risk, an acceptable (ie 'safe') dose has been considered by some regulatory authorities to lie in the probability range between 10^{-5}–10^{-7}. Daily exposure of a population to that 'safe' dose for a life time would be expected to increase the incidence of cancer by between one person in 10^5 to one in 10^7. Risks of this magnitude, which would result in cancer incidences that are much below those that would be detected by epidemiological methods, have been referred to as 'virtually safe'. Such an increase may not seem significant in terms of the range of the other risks to the general population (eg from motor accidents), but should be considered for an individual in relation to their other risk factors for that particular end-point (eg cancer). Furthermore, with carcinogens there may be a direct dose response relationship for the risk. For example, the additional risk of cancer from exposure to a particular dose of a carcinogen may be 1 in 10,000. If one million people receive such a dose then 100 extra deaths from cancer may result in that population. At present in the UK the risk of dying of cancer is 1 in 4 (25%) and this dose will add to it an additional risk of 1 in 10,000, raising the risk by just 0.01% to 25.01%.

Although the preceding mathematical extrapolations have been used for estimating a safe exposure using only animal data, risk assessment should incorporate human data whenever possible. Indeed human data may sometimes be used to demonstrate that cancer risk extrapolations are incorrect. Experience of this kind emphasises the importance of sound overall judgement when making quantitative risk assessments for a given chemical and for caution when using mathematical models which rely exclusively on animal data. Such models rest upon assumptions which are unproven and, on occasions, are biologically implausible or naive.

Limitations in mathematical estimation of risk

The preceding chapters have explored the complexity of the events which occur between exposure to a chemical carcinogen and the induction of a neoplasm. In particular the complexities of absorption, transport in the body, metabolism and the occurrence of the ultimate carcinogen in the susceptible organ have been noted, as has the increasing understanding of the way in which damage to DNA produces sequential mutations in the genome leading to cancer. Differing

capacities of the repair processes in different organs and species further complicates the picture. The models which have been used most extensively (such as the multistage models) do not take account of these complexities and instead make a number of assumptions, some of which may be incorrect for particular carcinogenic chemicals or responses. Examples of these assumptions are:

(i) that the time taken to induce cancer is proportional to the expected lifetime of the species (70 years in humans, 2 years in rodents);

(ii) that the duration of the administration of the chemical should be extrapolated linearly between species although it is known that cancer incidence increases proportional to the third or fourth power of the duration of exposure;

(iii) that the dose-response is linear and without threshold at low doses in spite of different repair capacities;

(iv) that the tissue dose of the ultimate carcinogen can be calculated as an expression of the body weight of the species under consideration;

(v) and that organ specificity, which often differs between species, is quantitatively unimportant in risk assessment.

There are many other ways in which the simplifying assumptions used in modelling can be misleading. Evidence that this is so is strongest when comparing risk assessments from different models using the same experimental data (Figure 7.2). The widely different extrapolation at low doses is due to the different assumptions used in the models and suggests that the results of these methods of risk assessment should be used with caution.

A quantitative risk assessment generates a numerical measure of the risk, whilst a qualitative risk assessment compares the hazard in terms relative to other situations. The Committee does not support the routine use of quantitative risk assessment for chemical carcinogens. This is because the present models are not validated, are often based on incomplete or inappropriate data, are derived more from mathematical assumptions than from a knowledge of biological mechanisms and, at least at present, demonstrate a disturbingly wide variation in risk estimates depending on the model adopted.

Risk evaluation and other considerations

The Committee on Carcinogenicity evaluates data on a case-by-case basis, taking into account the weight of all the available evidence. The range of data considered may differ with circumstances, for instance, it will not always be possible to obtain epidemiological data; and each assessment will be considered on its own merits. It is not possible to provide a universally applicable list of data which will be needed for a carcinogenicity risk assessment.

The Committee carries out qualitative evaluations of carcinogenic risks associated with chemicals. It does not provide quantitative estimations of risk (as

strictly defined). The Committee is not asked to comment on the needs for (or benefits expected from) the chemicals concerned, as advice on these aspects and risk-benefit judgements generally fall outside its remit. The Committee may, however, occasionally offer informal comments on these aspects. In all cases, the role of the Committee is to provide advice on a non-statutory basis and it is for the Government regulatory agencies concerned to decide whether such advice can be accepted in its entirety, and how it should be implemented. Government also receives advice from other expert committees which are concerned with risk-benefit aspects.

In assessing the acceptability of a risk it is important to take account of the public's perception of that risk. The risks that result from voluntary activity, such as cigarette smoking, tend to be perceived as more acceptable than those from involuntary or 'invisible' causes, such as exposure to pollution. As another example, a risk from something intentionally added to food is generally regarded as being less acceptable than a similar risk to health from a natural constituent of the diet. Any risk associated with the use of a chemical needs to be balanced against the health or economic benefits associated with that use and the availability of alternatives.

Conclusion

It is possible to establish threshold doses in animal studies for many of the toxic effects of chemicals, including the induction of cancer by some non-genotoxic carcinogens. These data can then be used in the safety evaluation for human safety limits. By contrast it is prudent to assume that for genotoxic carcinogens there is no threshold dose. This approach, together with the practical difficulties of using low doses in animal carcinogenicity studies, has led to the development of mathematical models that attempt to provide a 'best estimate' of the likely extrapolation of the dose-response curve below the lowest experimental data points, which are taken almost exclusively from animal bioassays. These models may give an impression of precision which cannot be justified from the approximations and assumptions upon which they are based. They are less persuasive than the broadly based approach to assessing putative carcinogens adopted by the Committee on Carcinogenicity which uses all the available data and which draws on expertise and information from a wide range of medical and scientific opinion.

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GLOSSARY

NOTE: *Some of the terms commonly used in these Guidelines—'carcinogen', 'tumour', 'genotoxic'—are extremely difficult to define in any formal manner. The entries in this section should, therefore, be regarded primarily as descriptive and explanatory.*

ABSOLUTE RISK Usually this term means the observed or calculated risk of an event in a population under study, as contrasted with the relative risk (qv). Sometimes, however, it is a synonym for attributable fraction, excess risk or risk difference; because of the inconsistency, the term 'absolute risk' should be avoided. (See also 'relative risk').

ADDUCT A chemical moiety which is covalently bound to a large molecule such as DNA or protein.

ALKYLATING AGENT Chemicals which are electrophilic reactants that, without the need for metabolic activation, leave an alkyl group covalently bound to a nucleophilic centre (mainly sulphur, nitrogen and oxygen atoms) in biologically important macromolecules such as proteins and nucleic acids. They are classified as mono-, bi- and polyfunctional depending on the number of reactive groups they contain. Many alkylating agents are mutagenic, carcinogenic and immunosuppressive.

ALLELES Alternative forms of a gene found at the same locus on homologous chromosomes.

ANEUPLOIDY The circumstances in which the total number of chromosomes within a cell is not an exact multiple of the normal haploid number. Chromosomes may be lost or gained during cell division, usually as a result of non-disjunction or anaphase lag.

ANTI-ONCOGENE See 'tumour suppressor gene'.

BIAS An inference which at any stage of an epidemiological investigation tends to produce results that depart systematically from the true values (to be distinguished from random error). The term does not necessarily carry an imputation of prejudice or any other subjective factor such as the experimenter's desire for a particular outcome.

CANCER Synonym for a malignant neoplasm—that is, a tumour that grows progressively, invades local tissues and spreads to distant sites. (See also 'tumour', 'invasion', 'metastasis').

CARCINOGENESIS The origin, causation and development of tumours. The term applies to all forms of tumours, benign as well as malignant and not just to carcinomas (cf 'tumorigenesis').

CARCINOGENS The causal agents which induce tumours. They include exogenous factors (chemicals, physical agents, viruses) and endogenous factors such as hormones. Chemical carcinogens are structurally diverse and include naturally-occurring substances as well as synthetic compounds. An important distinction can be drawn between *genotoxic* carcinogens which have been shown to react directly with and mutate DNA, and *non-genotoxic* carcinogens which act through other mechanisms. The activity of genotoxic carcinogens can often be predicted from their chemical structure—either of the parent compound or of activated metabolites. Most chemical carcinogens exert their effects after prolonged exposure, show a dose-response relationship and tend to act on a limited range of susceptible target tissues. Carcinogens are sometimes species- or sex-specific and the term should be qualified by the appropriate descriptive adjectives to aid clarity. Several different chemical and other carcinogens may interact, and constitutional factors (genetic susceptibility, hormonal status) may also contribute, emphasising the multifactorial nature of the carcinogenic process.

CARCINOMA Malignant tumour arising from epithelial cells lining (for example) the alimentary, respiratory and urogenital tracts and from epidermis, also from solid viscera such as the liver, pancreas, kidneys and some endocrine glands. (See also 'tumour').

CASE-CONTROL STUDY (Synonyms—case comparison study, case referent study) A study that starts with the identification of persons with the disease of interest and a suitable control group of persons without the disease. The relationship of some attribute to the disease (such as occupational exposure to a carcinogen) is examined by comparing the diseased and nondiseased with regard to how frequently the attribute is implicated in each of the groups.

CELL The unit of all living organisms and the smallest component which is capable of independent survival. In multicellular organisms they form tissues and organs. Normal cells vary greatly in their structural and functional organisation but, in general terms, they have three basic features—a defining outer membrane; biochemical components which support cellular growth, differentiation and repair; and genetic machinery which enables them to reproduce. All three components are aberrant in tumour cells.

CHROMOSOME In simple prokaryotic organisms such as bacteria and most viruses, the chromosome consists of a single circular molecule of DNA containing the entire genetic material of the cell. In eukaryotic cells the chromosomes are thread-like structures, composed mainly of DNA and protein, which are present within the nuclei of every cell. They occur in pairs, the numbers varying from one to more than 100 per nucleus in different species. Normal somatic cells in humans have 23 pairs of chromosomes, each consisting of linear sequences of DNA which are known as genes (qv). Chromosomes in tumour cells are frequently abnormal in terms of structure and/or number.

CLASTOGEN An agent that produces chromosome breaks and other structural aberrations such as translocations. Clastogens may be viruses or physical agents

as well as chemicals. Clastogenic events play an important part in the development of some tumours.

COHORT STUDY (Synonyms—follow-up, longitudinal, prospective study) The method of epidemiological study in which subsets of a defined population can be identified who may be exposed to a factor or factors hypothesized to influence the probability of occurrence of a given disease. An essential feature of the method is observation of the population for a sufficient number of person-years to generate reliable incidence or mortality rates in the population subsets. This generally implies study of a large population and/or study for a prolonged period of time.

CONFIDENCE INTERVAL, CONFIDENCE LIMITS A range of values determined by the degree of presumed random variability in a set of data, within which the value of a parameter (eg a mean) is thought to lie, with the specified level of confidence. The boundaries of a confidence interval are the confidence limits.

CONFOUNDING VARIABLE (Synonym—confounder) A factor that distorts the apparent magnitude of the effect of a study factor on risk. Such a factor is a determinant of the outcome of interest and is unequally distributed among the exposed and the unexposed; it must be controlled for in order to obtain an undistorted estimate of a given effect.

COVALENT BINDING Chemical bonding formed by the sharing of an electron pair between two atoms. Molecules are combinations of atoms bound together with covalent bonds.

DIFFERENTIATION A term that denotes the degree of morphological and functional organisation within cells and organs. Differentiation is implicit in normal histogenesis and organogenesis; it is to a varying extent aberrant in neoplastic cells.

DNA (DEOXYRIBOSENUCLEIC ACID) The carrier of genetic information for all living organisms except the group of RNA viruses. Each of the 46 chromosomes in normal human cells consists of 2 strands of DNA containing up to 100,000 nucleotides, specific sequences of which make up genes (qv). DNA itself is composed of two interwound chains of polynucleotides, each nucleotide consisting of 3 elements: a pentose sugar, a phosphate group and a nitrogenous base derived from either purine (adenine, guanine) or pyrimidine (cytosine, thymine).

ECOLOGICAL OR CORRELATION STUDIES Epidemiological investigations in which various measures of the characteristics of a population are contrasted with measures of disease. These paired observations are contrasted over differing circumstances (eg geographical or social) in an attempt to learn more of the interrelationship between the disease in question and the population variables.

ELECTROPHILE Compound containing an electron-deficient centre which tends to acquire electrons during chemical reactions—for example, an ultimate carcinogen forming adducts with DNA.

EPIDEMIOLOGY Study of the distribution and, in some instances, the causal factors of disease in communities and populations. Originally confined to infectious diseases—epidemics—but now increasingly applied to non-infectious conditions such as cancer. Important sources of epidemiological information are derived from incidence and mortality data for specified tumour types, usually expressed as rates, and from ecological, case-control and cohort studies (qv).

GAMETE A reproductive cell (ovum or sperm) which normally contains a haploid number of chromosomes.

GENE The functional unit of inheritance: a specific sequence of nucleotides along the DNA molecule, forming part of a chromosome (qv).

GENE AMPLIFICATION The process by which the number of copies of a specific gene within a cell can be increased. This usually confers a selective advantage on a cell—for example, in circumstances where increasing the copy-number of a gene can increase the rate of cell proliferation. Some oncogenes are amplified during carcinogenesis.

GENOTOXIC The ability of a substance to cause DNA damage, either directly or after metabolic activation.

GENOTYPE The genetic constitution of an individual.

HAZARD The intrinsic capacity of a chemical substance to cause an adverse effect on health.

HYPERPLASIA An increase in the size of organs and tissues due to an increase in the total numbers of the normal cell constituents.

INITIATION (See 'tumour initiation')

INTERVENTION STUDIES Epidemiological investigations, usually of a prospective cohort design, where a proportion of subjects in the cohort have had a procedure applied to them in the light of some knowledge which it is thought will decrease disease occurrence in those participants. In chronic disease, especially cancer, this type of study is rare because a successful outcome is unlikely for very many years.

INVASION The process whereby malignant tumours infiltrate through local tissues, often damaging or destroying them. (Benign tumours, by contrast, do not invade surrounding structures although they may compress them). A distinction is drawn between invasion and metastasis (qv) in which malignant tumour cells disseminate to *distant* sites.

LEUKAEMIA A group of neoplastic disorders affecting blood-forming elements in the bone marrow, characterised by uncontrolled proliferation and disordered differentiation or maturation. Examples include the lymphatic leukaemias which develop from lymphoid cells and the myeloid leukaemias which are derived from myeloid cells.

LYMPHOMA Malignant tumours arising from lymphoid tissues. They are usually multifocal, involving lymph nodes, spleen, thymus and sometimes bone

marrow and other sites outside the anatomically defined lymphoid system. (See also 'tumour').

METASTASIS The process whereby malignant cells become detached from the primary tumour mass, disseminate (mainly in the blood stream or in lymphatics) and 'seed out' in distant sites where they form secondary or metastatic tumours. Such tumours tend to develop at specific sites and their anatomical distribution is often characteristic; it is non-random. The capacity to metastasise is the single most important feature of malignant tumours.

MICROSOMES Subcellular particles of endoplasmic reticulum obtained from homogenized cells. They are rich in metabolising enzymes such as the cytochrome P-450 mixed function mono-oxygenases and certain conjugating enzymes.

MITOGEN A stimulus which provokes cell division in somatic cells.

MULTISTAGE CARCINOGENESIS The development of tumours in humans and animals is regarded as a multistage process in which both genotoxic and non-genotoxic changes occur. 3 separate phases—initiation, promotion and progression (qv)—have been described. Previously held ideas about these phases are, however, being modified in the light of current knowledge and additional stages may be defined in the future.

MUTATION A permanent change in the amount or structure of the genetic material in an organism which can result in a change in the phenotypic characteristics of the organism. The alteration may involve a single gene, a block of genes, or a whole chromosome. Mutations involving single genes may be a consequence of effects on single DNA bases (point mutations) or of large changes, including deletions, within the gene. Changes involving whole chromosomes may be numerical or structural. A mutation in the germ cells of sexually reproducing organisms may be transmitted to the offspring, whereas a mutation that occurs in somatic cells may be transferred only to descendent daughter cells.

NECROSIS Irreversible damage of cells; cell death.

NUCLEOPHILE An atom or group of atoms that tends to donate an electron pair to an electron-deficient (electrophilic) region of a molecule. DNA contains many nucleophilic centres.

ONCOGENE The name given to activated forms of proto-oncogenes (qv). They are cellular in origin.

ORGANELLES Small persistent structures with specialised functions which occur in individual cells: they are broadly analogous to the individual organs of complete multicellular organisms. Nuclear organelles include nucleoli, chromatin and centrioles. Cytoplasmic organelles include mitochondria, lysosomes, peroxisomes, ribosomes and endoplasmic reticulum. Most organelles are too small to be seen by conventional light microscopy and their structure is only apparent when viewed in the electron microscope.

PEROXISOMES Subcellar cytoplasmic particles bound by a single membrane. Probably present in all eukaryotic and possible some prokaryotic cells, but

seemingly more abundant in liver and kidney. They contain the enzymes of a β-oxidation system, the function of which is not entirely clear, but which generates hydrogen peroxide. This is normally detoxified by the peroxisomal enzyme, catalase. In some species hepatic and, to a lesser extent, renal peroxisomes may undergo proliferation in response to certain physiological conditions or as a result of treatment with various chemicals ('peroxisome proliferators'). The rodent liver and kidney is particularly susceptible to these effects whereas primates, including humans, appear to be refractory.

PHARMACODYNAMICS Studies of the effects of drugs on the body and the underlying modes of drug action.

PHARMACOKINETICS The description of the fate of drugs in the body, including a mathematical account of their absorption, distribution, metabolism and excretion. For compounds other than drugs, the corresponding terms should properly be 'toxicokinetics' although some toxicologists use 'pharmacokinetics' to cover all types of compounds. (Similarly 'pharmacodynamics' and 'toxicodynamics').

PHENOTYPE The observable physical, biochemical and physiological characteristics of a cell, tissue, organ or individual, as determined by its genotype and the environment in which it develops.

PROCARCINOGEN An inactive carcinogen which is metabolically converted, via proximate carcinogens, to the electrophilic ultimate carcinogen that reacts with DNA.

PROGRESSION (See 'tumour progression').

PROMOTION (See 'tumour promotion').

PROPORTIONATE MORTALITY RATIO (PMR) The ratio of the number of deaths from a given cause in a specified time period, per a unit number of total deaths in the same time period. Can give rise to misleading conclusions if used to compare mortality experience of populations with different distributions of causes of death.

PROTO-ONCOGENE A group of normal cellular genes, highly conserved, which are concerned with the control of cellular proliferation and differentiation. They can be activated in various ways to forms which are closely associated with one or more steps in carcinogenesis. Mechanisms of activation include point mutations which alter the structure of the proto-oncogene, or changes in the regulatory regions which alter the level of expression. Activating agents include chemicals and viruses. The process of proto-oncogene activation is thought to play an important part at several stages in the development of tumours.

PROXIMATE CARCINOGENS Metabolites, generated from procarcinogens, which in turn give rise to ultimate carcinogens.

RELATIVE RISK The ratio of the risk of disease or death among the exposed to the risk among the unexposed; this usage is synonymous with risk ratio. Alternatively, the ratio of the cumulative incidence rate in the exposed to the cumulative incidence rate in the unexposed, ie the cumulative incidence ratio. (See also 'absolute risk').

RISK A technical term (cf common usage) which is used to indicate the probability of an adverse health effect such as cancer developing in a human population within a defined set of circumstances. It is essentially a quantitative and statistical concept. Quantitative risk assessment of chemical carcinogens presents several problems at present, both in terms of the validity of the mathematical analyses used to predict cancer-risk and the accuracy of the results that are obtained.

SARCOMA Malignant tumour arising from connective tissues such as fat, cartilage or bone. (See also 'tumour').

SECULAR TREND (Synonym—temporal trend). Changes over a long period of time, generally measured over years or decades. Examples in the United Kingdom in this century include the decline of stomach cancer mortality and the rise, followed by the recent, slow decline, in lung cancer mortality among men.

SENSITIVITY AND SPECIFICITY (of a screening test) Sensitivity is the proportion of truly diseased persons in the screened population who are identified as diseased by the screening test. Specificity is a measure of the probability of correctly diagnosing a case, or the probability that any given case will be identified by the test.

SOMATIC Occurring in cells of the body, other than germ cells (See 'Mutation').

STANDARDISED MORTALITY RATIO (SMR) The percentage of the number of deaths observed in the study population to the number of deaths expected if it had the same age structure as the standard population.

STATISTICAL SIGNIFICANCE Statistical methods allow an estimate to be made of the probability of the observed (or greater) degree of association between independent and dependent variables under the null hypothesis. From this estimate, in a sample of given size, the statistical 'significance' of a result can be stated. The level of statistical significance is usually stated by the p Value.

THRESHOLD The lowest dose which will produce a toxic effect and below which no toxicity is observed.

TOXICOKINETICS (See 'pharmacokinetics')

TRANSFORMATION The process by which a normal cell acquires the capacity for neoplastic growth. Complete transformation occurs in several stages both *in vitro* and *in vivo*. One step which has been identified *in vitro* is 'immortalisation' by which a cell acquires the ability to divide indefinitely in culture without undergoing senescence. Such cells do not have the capacity to form tumours in animals, but can be induced to do so by extended passage *in vitro*, by treatment with chemicals, or by transfection with oncogene DNA. The transformed phenotype so generated is usually, but not always, associated with the ability of the cells to grow in soft agar and to form tumours when transplanted into syngeneic animals. It should be noted that each of these stages of transformation can involve multiple events which may or may not be genetic. The order in which these events take place, if they occur at all, *in vivo* is not known.

TUMOUR (Synonym—neoplasm) A mass of abnormal, disorganised cells, arising from pre-existing tissue, which are characterised by excessive and uncoordinated proliferation and by abnormal differentiation. BENIGN tumours show a close morphological resemblance to their tissue of origin; grow in a slow expansile fashion; and form circumscribed and (usually) encapsulated masses. They may stop growing and they may regress. Benign tumours do not infiltrate through local tissues and they do not metastasise. They are rarely fatal. MALIGNANT tumours (synonym—cancer) resemble their parent tissues less closely and are composed of increasingly abnormal cells in terms of their morphology and function. Well differentiated examples still retain recognisable features of their tissue of origin but these characteristics are progressively lost in moderately and poorly differentiated malignancies: undifferentiated or anaplastic tumours are composed of pleomorphic cells which resemble no known normal tissue. Most malignant tumours grow rapidly, spread progressively through adjacent tissues and metastasise to distant sites. Tumours are conventionally classified according to the anatomical site of the primary tumour and its microscopical appearances, rather than by aetiology. Some common examples of nomenclature are as follows: Tumours arising from epithelia: *benign*—adenomas, papillomas; *malignant*—adenocarcinomas, papillary carcinomas. Tumours arising from connective tissues such as fat, cartilage or bone: *benign*—lipomas, chondromas, osteomas; *malignant*—liposarcomas, chondrosarcomas, osteosarcomas. Tumours arising from lymphoid tissues are malignant and are called lymphomas; they are often multifocal. Malignant proliferations of bone marrow cells are called leukaemias. Benign tumours may evolve to the corresponding malignant tumours; examples involve the adenoma → carcinoma sequence in the large bowel in humans, and the papilloma → carcinoma sequence in mouse skin.

TUMOUR INITIATION A term originally used to describe and explain observations made in laboratory models of multistage carcinogenesis, principally involving repeated applications of chemicals to the skin of mice. Initiation, in such contexts, was the first step whereby small numbers of cells were irreversibly changed, or initiated. Subsequent, separate events (see 'tumour promotion') resulted in the development of tumours. It is now recognised that these early , irreversible, heritable changes in initiated cells were due to genotoxic damage, usually in the form of somatic mutations and the initiators used in these experimental models can be regarded as genotoxic carcinogens.

TUMOUR PROGRESSION The phase in the carcinogenic process when tumours acquire one or both of the pathognomic features of malignant growth—the capacity to invade local tissues and to disseminate to distant sites (metastasis). Progression is difficult to appraise in humans where tumours arising at many sites appear to be malignant without an identifiable preceding benign phase. An important example of progression is, however, provided by the adenoma → carcinoma sequence observed in the large intestine. The mechanisms of tumour progression are obscure, but they appear to include both genotoxic and non-genotoxic events.

TUMOUR PROMOTION An increasingly confusing term, originally used, like 'tumour initiation', to describe events in multistage carcinogenesis in exper-

imental animals. In that context, promotion is regarded as the protracted process whereby initiated cells undergo clonal expansion to form overt tumours. The mechanisms of clonal expansion are diverse, but include direct stimulation of cell proliferation, repeated cycles of cell damage and cell regeneration and release of cells from normal growth-controlling mechanisms. Initiating and promoting agents were originally regarded as separate categories, but the distinction between them is becoming increasingly hard to sustain. The various modes of promotion are non-genotoxic, but it is incorrect to conclude that 'non-genotoxic carcinogen' and 'promoter' are synonymous.

TUMOUR SUPPRESSOR GENE (Synonym—anti-oncogene, recessive oncogene) A gene whose continued expression is thought to be essential for normal growth and differentiation of cells. Many tumour suppressor genes probably exist, deletion or suppression of which appears to be a critical event in tumour development.

ULTIMATE CARCINOGEN The reactive (electrophilic) form of a carcinogen which forms adducts with DNA.

UNSCHEDULED DNA SYNTHESIS (UDS) DNA synthesis that occurs at some stage in the cell cycle other than the S period (the normal or 'scheduled' DNA synthesis period) in response to DNA damage. It is usually associated with DNA repair.

Appendix: Terms of reference for the Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment

1. At the request of:

Department of Health
Ministry of Agriculture, Fisheries and Food
Department of the Environment
Department of Trade and Industry
Department of Transport
Department of Energy
Health and Safety Executive
Medicines Commission, Section 4 Committees and the Licensing Authority
Committee on the Medical Aspects of Food Policy
Home Office
Scottish Home and Health Department
Department of Agriculture and Fisheries for Scotland
Welsh Office
Department of Health and Social Services for Northern Ireland
Other Government Departments

to assess and advise on the carcinogenic risk to man of substances which are:

a. used or proposed to be used as food additives, or used in such a way that they might contaminate food through their use or natural occurrence in agriculture, including horticulture or veterinary practice, or in the distribution, storage, preparation, processing or packaging of food;

b. used or proposed to be used in the treatment of drinking water in such a way that they might be present in the water supply;

c. used or proposed to be used or manufactured or produced in industry, agriculture, food storage or any other workplace;

d. used or proposed to be used in household goods, toiletries, cosmetics etc;

e. used or proposed to be used as drugs, when advice is requested by the Medicines Commission, Section 4 Committees, or the Licensing Authority; or

f. used or proposed to be used or disposed of in such a way as to result in the pollution of the environment.

2. To advise on important general principles or new scientific discoveries in connection with carcinogenic risks, to co-ordinate with other bodies concerned with the assessment of carcinogenic risk, and to present recommendations for carcinogenicity testing.

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