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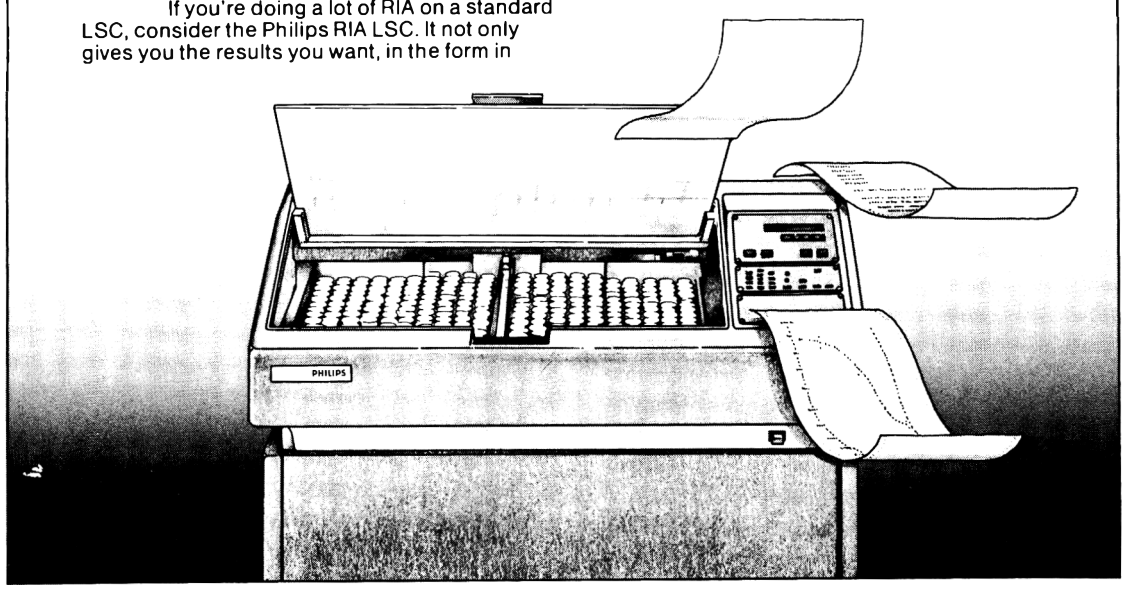

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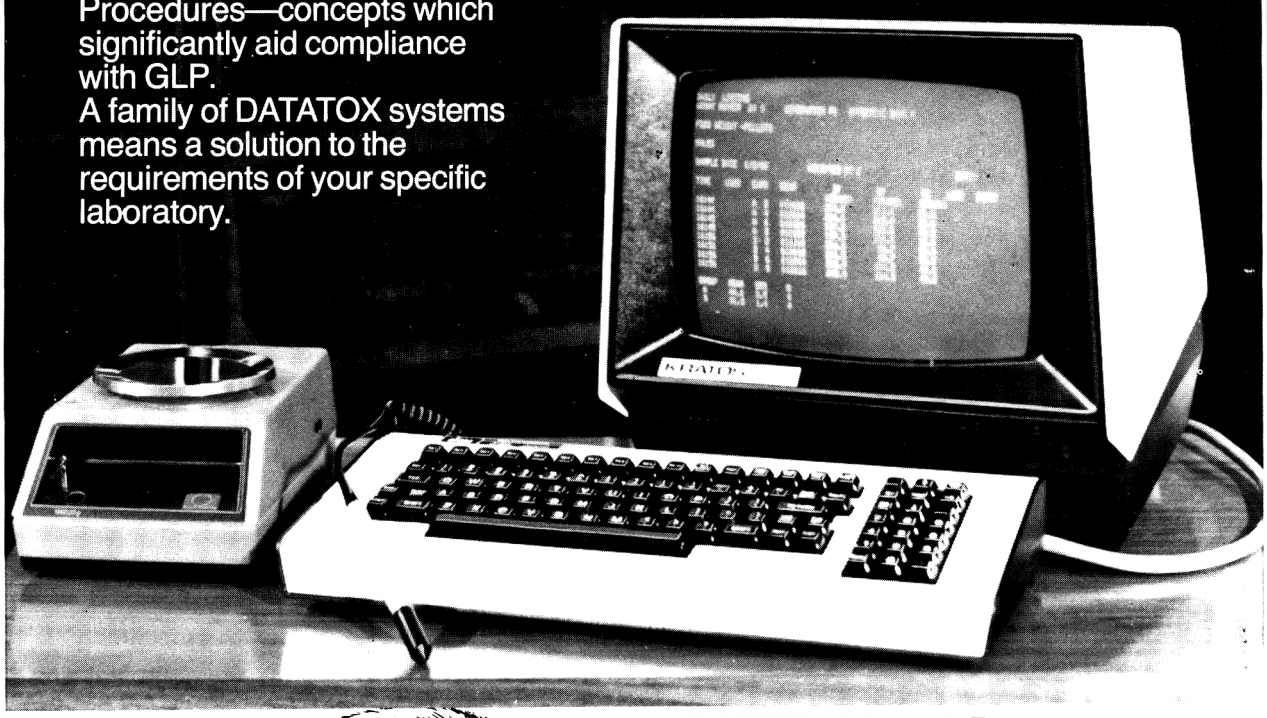

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- To promote food science and technology
- To stimulate investigation into the problems of food science and technology and to provide a medium for publishing and discussing the results of such investigations
- To provide a means for the free interchange of information and ideas in the English and French languages among food scientists and technologists
- To promote recognition of the role of people engaged in the field of food science and technology
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Leon Golberg MB, DSc, DPhil, FRIC, FRCPath
Founder-Editor of *Food and Cosmetics Toxicology*
First Director of BIBRA 1961–1967

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TWENTY YEARS OF TOXICOLOGY

A Special Issue dedicated to Leon Golberg

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TWENTY YEARS OF TOXICOLOGY

This volume is intended to commemorate the retirement of Dr Leon Golberg from his active role of President of a research establishment in toxicology—namely, the Chemical Industry Institute of Toxicology in the USA—and to remember his seminal contribution to the establishment of BIBRA, which he directed on its inception in 1961. It is not intended to present here a review of Dr Golberg's contribution to toxicological science—such would not be possible in so slender a tome—but rather to illustrate and emphasize the motive forces that have dominated his career to date by a collection of reviews from friends and colleagues touching on the wide-ranging scientific interests with which he has been associated and which have been so evident in the spectrum covered by toxicology during the twenty years of BIBRA's existence.

Throughout this period two problems have dominated toxicological thought—hepatic function and carcinogenesis—and these amply illustrate both the variable nature of scientific progress and the difficulties that face the toxicologist in reconciling new science and social consequences.

Consideration of hepatic function has related almost exclusively to the mixed-function oxidases and the analytical identity of the associated cytochromes. The period has been characterized by a tremendous output of technical data which have served to define in increasing detail the properties and conditions of activity of the component enzymes and their specificities. Although this represents unquestionably an impressive advance, there appears to have been no substantial increase in our understanding of the fundamental control of the hepatocyte response or of what determines that response. The intimate characterization of biochemical processes is obviously essential, but the time is ripe for an inspired leap, perhaps into the dark, to explain the exquisite versatility of the enzyme systems so far characterized, and to relate the changes in their function to the morphological appearances so beloved by pathologists.

It is possible, of course, that the obsession with the mixed-function oxidases has inhibited consideration of other microsomal systems, and current work with microsomal monoamine oxidases suggests that they have a potent role in the metabolism of some nitrosamines. By the same token, the biochemists' concentration on the smooth endoplasmic reticulum (or what passes for it in their hands) has resulted in the relative neglect of other organelles, and the current perception of the reactivity of the peroxisome promises exciting developments in the next two decades. Let us hope that the biochemists to whom this task falls will exhibit rather more intellectual breadth and versatility than their tutors and that they have the time to exploit it.

The concept of chemical mutagenesis and carcinogenesis has flourished beyond all expectation in the last twenty years. This has been due essentially to the development of relatively simple techniques to determine the ability of a chemical to interact irreversibly with DNA in an *in vitro* model, and to the remarkable progress in identifying the enzyme systems involved in effecting the changes dictated by DNA normally and after modification. Again, the dominance of technology (as opposed to science) has resulted in a considerable increase in the numbers of models available for study, and there has, as yet, been but little attempt to elucidate and characterize the mechanisms involved. This approach seems to have progressed only slightly beyond the concept of methylation of DNA, a process which itself has been by no means conclusively demonstrated to have a causal role in mutagenesis or carcinogenesis. It must surely be the case that a precise study of the chemical action of DNA poisons will throw light on the specificity of *in vitro* models and, more importantly, on the ability of mammalian systems to effect repair. At the same time it is necessary to ensure that the notion of 'epigenetic' carcinogenesis is not allowed to masquerade as a solution merely because it expresses the problem eloquently.

There has been relatively little progress in a number of areas, though some of these are now becoming fashionable and will perhaps move forward in the near future. Reproductive toxicology has been particularly punished by precept unsupported by fact and, given the concern about hormonal and other effects in the second generations, the field is ready for imaginative exploitation. Another field already undergoing such exploitation with, alas, insufficient factual input is the formalized approach to structure–activity relationships. The remarkable expansion of *in vitro* methodology allied to the characterization of chemicals generating reproducible effects must surely result in much progress in the near future, something that Dr Golberg has been very active in stimulating.

Finally, what is to be said of the regulatory scene? Leon Golberg has always been staunch in his criticism of the facile use of half-baked ideas for regulatory convenience, but despite his efforts the past two decades have seen this process carried to what would have seemed, twenty years ago, an unimaginable pitch. Much of it has been politically motivated. The notion that 90% of all cancer was environmentally determined was conveniently assumed by those who wanted to attack the chemical industry to mean that industrial chemicals in one form or another had a causal effect on cancer incidence in man. It has now come to mean that nine times out of ten a cell becomes cancerous because of some influence that arises outside itself—as far as can be determined an entirely unhelpful, and probably meaningless, concept.

A strong and steady improvement in the role and influence of the statistician has served to instruct the ignorant on the variability of the biological response, but, as yet, not a great deal more. The statistician's influence appears to be on the wane—possibly because it was never adequately allied to scientific initiative—and thus it is essential that, in the coming years, statistical analysis assumes its proper role in experimental design and is not allowed to undermine creative and intuitive thinking. Above all, it must not be allowed to impart a veneer of science to the barren approach of the regulator. The current vogue of 'risk assessment' is another manifestation of the same trend. Let no-one pretend that assessment of risk has any chance of success in the absence of some knowledge of toxic mechanisms; there never can be a circumstance in which the calculation of hazard can be more precise than the comprehension on which it is based.

The contributions to this volume cover many of these aspects and some speculate in the informed fashion of science. We have allowed our contributors more freedom than usual to present ideas that require substantiation because we intend to honour our Editor as a creative and imaginative thinker. There is still something of the pioneer in any toxicologist; and that, allied to a reverence for science, personifies Leon Golberg.

D. M. CONNING

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FCT—THE EARLY YEARS

In BIBRA's temporary office accommodation, barely a stone's throw from the hubbub of Piccadilly Circus, *Food and Cosmetics Toxicology* survived its embryonic existence and struggled to life in 1963. Its postnatal development was assured by the move to the rural tranquillity of Carshalton on completion of BIBRA's research laboratories in the following year.

Food and Cosmetics Toxicology was the brainchild of Dr Leon Golberg, BIBRA's first Director, who became the journal's Founder Editor. BIBRA became operational in 1961 and was set up to serve the interests primarily of the food and cosmetics industries—hence the title of the journal. But why a new journal? At that time very few specialist journals in toxicology existed, with the result that both notable and modest advances in this field were reported in diverse scientific and medical journals. Toxicological investigations had started to proliferate, as the expansion of the journal's research section was soon to testify, while the thalidomide tragedy and Rachel Carson's book *Silent Spring* provided further impetus to the growth of this multi-disciplinary science and its application to environmental, industrial and consumer safety.

Dr Golberg's vision was ambitious. He saw the journal not only as a medium for research scientists to publish their original findings on the safety evaluation of various chemical products, on fundamental advances in toxicity testing, on mechanisms of toxic action and on the interpretation of toxicological findings in animals in terms of human risk, but also as a means by which the non-specialist reader, such as the chemist or technologist working in industry, could keep abreast of world-wide developments in toxicology and legislative control. For this purpose an information section was to be specially prepared by BIBRA staff. Dr Golberg's own frequent and lively contributions to this section were often graced with much originality and wit—none more so than his classic indictment of the Delaney Amendment under the explicit title "When is a carcinogen not a carcinogen? When it is an essential nutrient". Similarly his decision to attach prominence in the information section to the early developments in the nitrosamine field has been vindicated; few others could have had the foresight to remark in 1963 that nitrosamines were "potentially one of the epoch-making developments in the history of cancer research".

Maintaining a balanced outlook on contentious issues has always been the aim of the journal—drawing attention to genuine risks and allaying unwarranted fears. Indeed the very first research paper in Volume 1 put into perspective alarmist claims of the hairspray thesaurosis risk. Soon afterwards encouragement was given to the publication of several teratogenicity studies on butylated hydroxytoluene, the negative results from which countered false alarms of an earlier, inadequately-designed study that had apparently demonstrated anophthalmia in the offspring of rats fed this food antioxidant.

The energies directed by Dr Golberg to the promotion of the journal continued unabated even after his departure to the USA in 1967, despite the demands first of Albany and then CIIT and his involvement with many national and international committees, the Presidency of the Society of Toxicology and so on. Mainly through his unstinted efforts, the journal has attracted many leading contributions from the world's centres of toxicological excellence. A further strengthening of international links may be expected from the recent establishment of a joint editorship, with Dr Golberg concentrating on contributions from the USA and Canada, and BIBRA's present Director, Dr David Conning, assuming responsibility for those from Europe, Africa, Asia, Australia and South America.

In his foreword to the first issue of *Food and Cosmetics Toxicology*, Dr A. J. Lehman, an outstanding pioneer in toxicology, wrote that if the objectives of the journal were fulfilled the journal would have a "long and useful life". This confident prediction has been realized and *Food and Cosmetics Toxicology* has established itself as a leading journal in toxicology, adapting to the changing emphasis of the science and the widening interests of BIBRA by extending its coverage to plastics, industrial and agricultural chemicals, environmental chemicals and certain pharmaceutical products.

One is reminded of the famous and often-quoted motto that adorned Dr Lehman's office in the Food and Drug Administration: "You too can learn pharmacology in two easy lessons, each ten years long". Readers of *Food and Cosmetics Toxicology*, whose loyalty has now extended to two decades, may feel that in toxicology two such lessons are just the beginning.

A. J. COHEN

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THE DELAYED LONG-TERM EFFECTS OF CHEMICALS FOLLOWING NEONATAL EXPOSURE IN LABORATORY ANIMALS

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(*Received 22 January 1981*)

Summary—The increased sensitivity in neonates to the effects of certain chemicals is attributable to the immaturity of detoxifying and excretory processes as well as to the interference of the chemical with postnatal development. An adverse effect of a chemical may appear in adulthood, even though it was induced in the postnatal period, because of the late development of the function affected, e.g. reproduction or slowly progressing pathological changes. Examples of neonatally acquired effects that have been transmitted to the next generation are presented. Adaptation of the protocol for reproduction toxicology studies is suggested for the detection of these delayed and persistent toxic effects.

Introduction

The premarketing safety assessment of non-medicinal products is based primarily on animal experiments. Generally the intended use of the test chemical influences the protocol for these studies; accordingly, a product to be marketed for infants should be tested in neonatal animals. Although legislation for preclinical safety studies of drugs in the United States was triggered in 1938 by an iatrogenic disease in children, the need for preclinical studies in neonatal animals was not recognized. The increasing amount of literature on neonatal pharmacology and toxicology indicates the current interest.

Perinatal pharmacology was the subject of earlier reviews (Cohlan, 1964; Done, 1966), but the possibility of delayed and long-term effects of chemicals after neonatal exposure was seldom considered. The discoloration of teeth and enamel hypoplasia induced by certain tetracycline antibiotics are the best-known examples in the clinical literature (Cohlan, 1964). The late behavioural effects of prenatal exposure to drugs and other chemicals are being investigated increasingly as a part of teratological studies in experimental animals, but there are few published studies of this nature in animals treated neonatally. This short review paper emphasizes the slowly developing, long-lasting adverse effects of xenobiotics in neonatal animals.

Unique sensitivity of neonates to xenobiotics

Infants are unavoidably exposed to potentially toxic chemicals from medicinal or hygienic products, food contaminants, air pollution and other sources, and may accumulate the xenobiotic because of a low rate of biotransformation or excretion. For example, chronic lead poisoning in toddlers is the result of cumulative low-level exposure from various materials, including house dust and food contaminants (Lin-Fu, 1973). The 'gray syndrome', a cardiovascular collapse in neonates treated with chloramphenicol, is the result of an insufficient rate of drug metabolism and excretion. In some instances, the lack of barriers to impede the penetration of xenobiotics into sensitive tissues, such as the brain and testicle, is responsible for the increased vulnerability of the newborn. Kernicterus, an encephalopathy, is the result of the displacement of bilirubin from its binding site in plasma by a chemical and the penetration of the bilirubin into the brain.

Deficient activity of various enzyme systems at the target site of a chemical can be responsible for the development of toxic effects. For example, erythrocytes of the newborn are very sensitive to oxidizing agents because of their low methaemoglobin-reductase activity.

The spectra of biological effects of chemicals in neonates may be different from those in the adult. The effect of a chemical can be qualitatively different in animals of various ages; for example clonidine, an antihypertensive drug, produces hypermotility in rats aged between 1 and 7 days and hypomotility in those older than 20 days (Nomura, 1980). Phenytoin has an excitatory effect in rats of less than 12 days of age and then an increasingly inhibitory effect on the central nervous system (CNS; Vernedakis & Woodbury, 1969). These differences are related to the gradual maturation of the interaction between various neural systems. An effect that occurs in the adult may not occur in the neonate; an indirectly acting sympathomimetic amine, for example, does not affect the heart of week-old rats because their synaptosomal amine-release system is not developed (Bareis & Slotkin, 1980).

When the mechanism of action of a chemical is related to an interference with development, the effect is a function of the time of administration. Such an effect is often a delayed one; it becomes evident at various times after the end of treatment and lasts for a long period or for a lifetime. Usually more than one mechanism is responsible for the unique sensitivity of the neonate to xenobiotics, since several co-existing variables (e.g. rate of metabolism, excretion, developmental state) influence the effect. For example, one of the first chemicals to which the newborn is exposed is usually an obstetric drug. The effects of obstetric analgesics on the newborn have been the subject of

numerous investigations during the last decades. In most of these studies, signs of transient CNS depression were detected (Aleksandrowicz, 1974). These effects are related to the increased biological half-life of the drug and to the great sensitivity of the newborn to CNS depressants. A delayed CNS depression may inhibit the acquisition of learned responses that are of adaptive significance even during the first weeks of life.

Delayed and long-term effects of neonatal treatment

The significance of early-life experiences for the development and adaptation of the organism has been demonstrated in behavioural studies in experimental animals. Rats handled frequently in the neonatal period had a smaller increase in plasma concentrations of adrenocortical steroids than non-handled rats when they were exposed to novel stimuli later in life (Levine, Haltmeyer, Karas & Denenberg, 1967). The habitat in which animals were reared affected their performance in adulthood (Denenberg & Rosenberg, 1967). Several chemicals given in large doses neonatally have a variety of delayed and long-lasting effects. An irreversible effect of certain CNS depressants on neuro-endocrine mechanisms affecting sexual development has been detected in hamsters, guinea-pigs and rats (Gorski, 1973). During the first week of life in these species, testicular androgen induces changes in the hypothalamus that are reflected later in the patterns of food intake, body weight, locomotor activity and sexual behaviour. Treatment of male hamsters with 100 μg pentobarbital on postnatal days 2–4 resulted in a significant decrease in their mating activities at 60 days of age compared with controls (Clemens, Popham & Ruppert, 1979). Data from other related studies are consistent with this finding, indicating that certain barbiturates interfere with the action of androgens (Gorski, 1973). A single injection of chlorpromazine (20 μg/g body weight) given to newborn male mice on postpartum days 1–15 inhibited testicular development assessed at 108 days of age (Hogarth & Chalmers, 1973).

Neonatal mice injected sc with monosodium glutamate (0·5–4 mg/g) developed neuronal necrosis in the hypothalamus. As adults they became obese and the females were infertile (Olney, 1969). *o,p'*-DDT given sc to newborn female rats in 20-mg doses for three consecutive days caused persistent oestrus and anovulation in adulthood (Gellert, Heinrichs & Swedloff, 1974).

Testicular androgens also affect—*via* the hypothalamic–pituitary pathway—the development of hepatic enzyme systems that metabolize endogenous steroids and xenobiotics. Postpubertal sex differences in the activities of these enzymes are both quantitative and qualitative. Exposure of neonatal male rats to various chemicals (e.g. diethylstilboestrol, polychlorinated biphenyls) that have oestrogenic activity produced irreversible changes (imprinting), leading to a female pattern of hepatic enzyme activity in their adulthood. The significance of such an event in the susceptibility to xenobiotics (e.g. to the hepatotoxicity of cadmium) has been demonstrated (Lui & Lucier, 1980). The result of such neonatal imprinting on the rate-determining enzymatic steps in cholesterol biosynthesis and degradation has also been shown; the fat and cholesterol contents of the diet of neonatal rats influenced the cholesterol homeostasis in adulthood (Naseem, Khan, Jacobson, Nair & Heald, 1980).

An alteration in the development of central neurotransmission has been postulated to occur in neonatal rats treated sc with 300 mg streptomycin/kg from postnatal day 2 to day 22 (Alleva & Balazs, 1978). These rats developed hyperactivity and a stereotypic dyskinesia consisting of circling, repetitive up and down head movements and backward gait. These effects lasted for several months. They were reversed by a single dose of dopaminergic agonists. Histological sections of the vestibular apparatus or brain showed no changes on light-microscopic examinations, but the dopamine content was altered in the caudate and accumbens nuclei and the serotonin receptors were altered in the frontal cortex. The syndrome did not develop when the treatment was begun in weaned rats with even higher doses of streptomycin.

Neonatal animals are also more sensitive to the ototoxic effects of aminoglycoside antibiotics than are young adults (Alleva & Balazs, 1980). Rats treated with 300 mg streptomycin sulphate/kg sc from postnatal day 2 to day 11 were deaf at the end of treatment. A similar dose given to rats from 25 to 45 days of age did not produce deafness. The ototoxic effect can be a delayed one; rats treated with streptomycin as above from postnatal day 2 to day 7 or day 8 to day 11 had normal hearing at days 11–14 but became deaf 2–3 wk later. In neonatally treated rats, streptomycin caused extensive cochlear lesions consisting of destruction of inner and outer hair cells in the organ of Corti, reduction of spiral ganglion cells and severe damage to the cochlear nerve.

Late-appearing adverse effects may occur following antineoplastic therapy in children. Osteoporosis after treatment with methotrexate and cardiomyopathy after anthracycline antineoplastic drugs have been reported (De Bernardi, 1980; Halazun, Wagner, Gaeta & Sinks, 1974). The mechanisms of the increased sensitivity of children to these effects are not known.

6-Mercaptopurine, an antineoplastic agent used in the therapy of leukaemia in children, has been found to produce a delayed and irreversible toxic effect in rats (Alleva, Balazs, Haberman, Weinberger & Slaughter, 1980). Daily sc treatment from 2 to 22 days of age with 2 mg 6-mercaptopurine/kg produced no clinically or histologically detectable effects at the end of treatment. However, at about 12 months of age, paresis of the hind legs was observed and histological examination showed marked atrophy of the lumbar and rear-leg muscles, although nerves and blood vessels were not affected. In a subsequent study (F. R. Alleva & T. Balazs, unpublished data 1980), the earliest histological changes in the muscle were seen 4 months after the end of treatment. Muscular atrophy was not detected in adult rats after chronic treatment with this drug. It is possible that the neonate is susceptible because the chance of interaction of the drug with muscle DNA and the persistence of the reaction are greater in the dividing muscle cell of the neonate than in the non-dividing cell of the adult.

The high rate of cellular replications may predispose the neonate to the effect of certain carcinogens.

In addition, the immaturity of the immune system could be a predisposing factor, for example in the great susceptibility of the neonatal lymphoreticular tissue to carcinogens. Leukaemia was produced in 21% of newborn mice given a single sc dose of 2 mg urethane, in 17% of 5-day-old mice given 4 mg and in 3% of 40-day-old mice given 20 mg (doses comparable on a mg/body surface area basis; Fiore-Donati, De Benedictis, Chieco-Bianchi & Maiorano, 1961). When urethane was injected ip into newborn mice weekly for 10 wk at a dose level of 1 mg/g, 35% developed lymphatic leukaemia at wk 60 compared with 6% of those given similar treatment from 45 days of age (Berenblum, Boiato & Trainin, 1966). Newborn mice lymphomas by dimethylbenzanthracene than were 2- or 4-wk-old mice (Toth, Rappaport & Shubik, 1963). A critical period was also found for the effects of various steroids; oestradiol-17β (5–20 μg) given to mice on postnatal days 1–5 produced in the vaginal epithelium permanent changes which developed to neoplasia in old age. These effects did not occur when treatment was started at 8–11 days of age (Takasugi, 1979).

Carcinogens that require metabolic transformation are expected to be less potent in the neonate since the activity of the drug-metabolizing enzyme system is at a low level.

Cross-generational effects following neonatal insults

Evidence from experimental animals indicates that some chemically induced changes in neonates are transmitted to the next generation. Some of these changes are attributable to mutation, but others may be due to an altered endocrine or metabolic state which persists in the mother and affects the foetus.

In addition to the persistent effects in the hypothalamus caused by the administration of androgens to neonatal rats and hamsters, other hormones (e.g. thyroxine) given to neonatal rats produce changes similar to those observed after destruction of the hypothalamic thyrotropic area. A total of 150 μg sodium *l*-thyroxine (T_4) given sc to rats during the first 5 or 7–10 days of life, or injection of systemically ineffective doses into the arcuate area of the hypothalamus, induced hypothalamo–pituitary, thyroid and gonadal abnormalities (Bakke, Lawrence, Robinson & Bennett, 1977). Hypothyroidism, delayed and irregular oestrus and subnormal responses to thyrotropin-releasing hormone and propylthiouracil were detected in these rats when they were 6–9 months of age. T_4 acted on certain developing hypothalamic centres, resulting in late and persistent effects. When the female rats treated neonatally with T_4 were mated with untreated males, their cross-fostered progeny had abnormalities in thyroid and gonadal functions. Some of these persisted into adult life and were present in the F_2 offspring.

Transmission of a chemically induced disorder in carbohydrate metabolism to the progeny of weanling rats treated with a single ip dose of 150 mg alloxan/kg has been demonstrated (Spergel, Kahn & Goldner, 1975). The treated rats had a decreased glucose tolerance throughout their lives. The presence of latent diabetes was detected in their untreated progeny and frank diabetes developed in the seventh generation. In this study and in the study described by Bakke *et al.* (1977), the entire population of the progeny was affected in varying degree.

A recent investigation in rats revealed that early weaning (at day 14) greatly increased their susceptibility to restraint-induced gastric erosion on postnatal day 27 (Skolnick, Ackerman, Hofer & Weiner, 1980). When prematurely weaned 3-month-old females were bred with males weaned at the usual time (day 21), their cross-fostered, normally weaned progeny also showed increased susceptibility to restraint-induced erosions. In addition, some of the behavioural changes brought about by the early environmental influences mentioned above have been shown to be transmitted to the progeny (Denenberg & Rosenberg, 1967). A behavioural change, an altered conditioned avoidance response, induced in rats by neonatal administration of neuroleptic drugs (e.g. trifluoperazine) occurred in their adulthood and also in their untreated cross-fostered offspring at 3 months of age (Gauron & Rowley, 1973).

Delayed and long-lasting effects on the immune system are expected to develop following neonatal exposure to antigenic chemicals. However, transmission of the acquired immune response to the progeny has been a recent discovery. A neonatally acquired and actively maintained state of antigen-specific tolerance to foreign major histocompatibility antigen in male mice was transmitted to the first and second generation offspring (Gorczynski & Steele, 1980).

Cross-generational effects of carcinogens have also been detected. Dimethylbenzanthracene given to mice during pregnancy and methyl- or ethylnitrosourea given to pregnant rats produced tumours not only in their progeny but also in the second and third generation (Tomatis, 1979).

Conventional theories have been challenged by data from these studies showing unexpected inheritance of a variety of chemically induced changes in the neonate. In some of the studies, the maternal environment alone could influence the development of the disorder, as in rats treated with thyroxine. Offspring of female rats thyroidectomized before mating also developed thyroid abnormalities, suggesting an altered set point of pituitary-thyroid regulation as a consequence of maternal hypothyroidism. In the breeding study with thyroxine-treated rats, female progeny were mated with normal males; thus the disorder was transmitted maternally (Bakke *et al.* 1977). A theory for the transfer of acquired metabolic abnormalities to the offspring involves an alteration in the cytoplasmic mitochondrial DNA present in the maternal egg, where it replicates and can later be found in the tissue of the progeny.

In the study with alloxan by Spergel *et al.* (1975), the males were also tested and found to be able to transmit the prediabetic condition to successive generations. This finding ruled out the role of an abnormal intrauterine environment or a diabetogenic milk factor induced by alloxan. The authors postulated that alloxan may permanently alter the function of regulator genes that, by diffusable products, regulate the genes directing glucose metabolism. This concept (polygenic inheritance) may explain the unimodal distribution of the effect as well as the increased severity of the glucose intolerance in successive generations. It may be speculated that such a mechanism (i.e. an

effect on regulator genes) also operates in the transmission of an acquired behavioural characteristic, such as the altered conditioned avoidance response induced by neuroleptics. It can be assumed that the behavioural characteristic is controlled by several genes under the control of regulator genes; for example trifluoperazine would alter the function of the regulator genes both in somatic and germ cells. This event is consistent with the finding that cross-breeding of drug-treated females and males does not seem to accentuate the effect obtained in drug-treated females.

In discussion of the mechanisms of cross-generational carcinogenesis, Tomatis (1979) postulated that the tumours occur (1) as a consequence of a mutation at a specific locus, resulting in cancer at a particular site or (2) following interaction with one or more environmental factors at a site and in individuals made more susceptible to neoplastic transformation by a heritable lesion which does not produce cancer by itself.

A process whereby somatic genes (normal or mutated) enter the germ line has recently been proposed as the mechanism for inheritance of acquired immunological tolerance (Gorczynski & Steele, 1980). None of these cross-generational effects has been investigated in classical genetic breeding studies. Further research efforts require confirmation of some of the findings and elucidation of the mechanisms.

Suggestions for premarketing safety studies in neonates

The examples of delayed effects of neonatal exposure to chemicals in experimental animals provide a background for experimental neonatal toxicology. Testing chemicals for delayed effects is indeed difficult. The metabolism of chemicals and the postnatal developmental stages of the various organ systems, and therefore the critical period for susceptibility to the chemical, vary among species. Whereas a certain developmental stage in an organ system may be reached in one species before birth, the same stage is attained postnatally in another species. For example, the sexual differentiation of behaviour occurs postnatally in most of the non-rodent laboratory animal species and prenatally in humans (Barraclough, 1967). For ethical reasons, comparative metabolic studies cannot be performed in human infants as an aid for the selection of the most appropriate experimental animal species. For all of these reasons, the use of more than one species of experimental animal is indicated. A few laboratories are using dogs and minipigs in current neonatal toxicity studies.

Performance of these studies appears to be in order when the test chemical is destined for use in women during late pregnancy (e.g. a drug to prevent premature labour) or during labour (e.g. an analgesic), or for use in infants (e.g. food additives, hygienic products). In addition, neonatal effects of certain industrial and environmental chemicals are of interest since lipophilic and slowly metabolized or excreted xenobiotics could be present in the milk of the nursing mother.

The performance of neonatal studies is laborious and expensive, and therefore an extension to the current teratological tests (perinatal/postnatal segment) would be a practical approach. In these current tests, animals are treated from the final trimester of gestation through weaning. The unique feature of the neonatal study is that the chronologically programmed development is monitored; for example, the times of eye and ear openings, dentition, appearance of specific reflexes and the first oestrus cycle in rats are determined. A representative number of the animals are killed at the end of treatment for haematological and gross and histopathological examinations, and the remaining animals are examined for signs of delayed effects.

The interpretation of findings in these tests in newborns can be more difficult than that in adult animals. The relevance to humans of some of the delayed effects in developing animals has not been established. For example, there are no reports of myopathy in children treated with 6-mercaptopurine or of streptomycin-induced hyperkinesis. Nevertheless these examples may represent more than biological curiosities. Extrapolation of the findings from one species to another is aided by an understanding of the mechanisms.

Research on neonatal carcinogenesis is of great significance, since cancer is second only to accidents as a cause of death in children. The peak incidence of leukaemia and lymphoma occurs at 4–6 years of age, suggesting that their induction occurs much earlier in life. Incorporation of the neonatal period in carcinogenicity tests has been considered and may obviate the need for a separate test.

In the safety evaluation of food additives, performance of multigeneration studies has been considered. This type of study—with appropriate modification—would be suitable for the detection of late as well as cross-generational effects. In human beings, delayed effects, as described above, are not readily attributable to neonatal exposure, with the possible exception of data from prospective epidemiological examinations. However, legal and ethical restraints restrict the performance of properly planned studies, making thorough investigation in experimental animals a necessity.

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RETHINKING THE ENVIRONMENTAL CAUSATION OF HUMAN CANCER

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Summary—Many of the hypotheses regarding the aetiology of cancer in man were suggested by the early 1950s and much recent effort has been directed to defining causes and mechanisms in more detail through more sophisticated epidemiological and laboratory approaches. Although there are many factors involved in its aetiology cancer may be prevented by controlling a single predominant factor. Epidemiology has contributed considerably to our understanding not only of the aetiological factors but also of the possible mechanisms involved, especially those associated with life-style. For cancers believed to be related to the latter, notably those of the gastro-intestinal tract and endocrine-dependent organs, there is evidence for the involvement of cultural, behavioural and dietary factors rather than direct exposures to carcinogens alone. Future efforts should be directed towards a better understanding of factors modulating carcinogenesis since it seems improbable at present that the initiating factors for many cancers can be identified.

Introduction

It is a great pleasure to contribute to this issue dedicated to Dr Leon Golberg. I have known Leon Golberg for many years, and admire not only his scientific work but also his erudite and objective studies on the complex interactions between toxicology and human health and welfare.

Present concepts of the aetiology of human cancer have developed gradually, reflecting new ideas and hypotheses arising from both epidemiological and laboratory studies. Thus, the isolation of pure carcinogenic polycyclic aromatic hydrocarbons was essentially dependent on the original observations of Percivall Pott. The later demonstration of other occupational hazards provided the foundations of modern chemical carcinogenesis. The recognition of oncogenic animal viruses and the development of pure strains of mice as our understanding of inherited susceptibility has increased has had considerable impact on aetiological theories. Willis (1948), while drawing attention to the possible environmental background of many human cancers, largely emphasized industrial chemical carcinogens. It is only comparatively recently that the role of environmental factors in human cancer in a wider sense has been accepted. Moreover it appears that progress in environmental carcinogenesis has been as much dependent on the confirmation or exclusion of hypotheses through more sophisticated investigations as on the promulgation of completely new theories.

In 1950, a group of distinguished experimentalists and laboratory workers meeting in Oxford (Clemmesen, 1950) concluded that geographical variations in cancer incidence predominantly reflected environmental influences. In that term, however, they included all exogenous factors which impinge on man, i.e. the dietary, social and cultural environments as well as discrete chemical carcinogens. Stimulated by the Oxford report, George Oettlé and myself decided to collect accurate statistics in Southern Africa to provide a baseline of cancer incidence in a newly urbanized population in an attempt to evaluate the relative aetiological importance of lifestyle, diet and industrialization. The results of this survey (Higginson & Oettlé, 1960) in combination with the migrant studies of Kennaway (1944), Haenszel (1961) and Haenszel & Kurihara (1968), and the reports of others (Boyland, 1969; Doll, 1967), strongly suggested that most human cancers were related to environmental factors and not to racial or hereditary factors (Higginson, 1960). Further, the survey suggested that "way of life" was of major significance for many cancers, especially those of the gastro-intestinal tract and endocrine-dependent organs. These estimates were later extended using a wider range of populations (Higginson, 1969).

Carcinogenesis in man

Definition of cause

The massive increase in the production and use of synthetic chemicals in industry, agriculture and medicine since 1940 caused renewed interest in the potential carcinogenic effects of chemical pollutants in the human environment (Carson, 1962). Since the concept of a discrete carcinogenic chemical, physical agent or virus as a direct cause of cancer was widely comprehended, the possibilities for prevention through simple legislative action were readily accepted. In contrast, there was a tendency to neglect the inherent complexities of chemical carcinogenesis, although such complexities and the many associated modulating factors involved had already been demonstrated by many workers (Berenblum, 1978; Clemmesen, 1950; Miller, E. C., 1978). Moreover, for many cancers in humans, the data were inconsistent with an aetiology based only on simple chemical exposures (Higginson & Oettlé, 1960). In addition, an increasing number of parameters related to life-style were identified and associated with increases or decreases in

cancer incidence. Such parameters, now called "carcinogenic risk factors", included absence of fibre in the diet, age at first marriage, etc., and could not readily be defined as carcinogens in the classical sense. Both laboratory and epidemiological studies, however, suggested that the role of such factors including their possible relationship to individual susceptibility should be explicable in more objective biological terms through a better understanding of the multistage and molecular basis of carcinogenesis.

The multifactorial nature of carcinogenesis tends theoretically to complicate the definition of cause, since, in addition to the role of complex modulating factors, combinations of carcinogens may show synergistic (Hammond & Selikoff, 1973) or inhibitory effects (Miller, Miller, Brown & MacDonald, 1958). In practice, however, a single factor may be so predominant as to be regarded as the practical cause in the public health sense, in that in its absence a significant proportion of related cancers would not arise. This in no way excludes a role for modulating factors (Cole & Merletti, 1980) or necessarily implies understanding of the basic mechanisms involved. Thus, although the action of cigarette smoking may be multiplied many times by asbestos exposure, the former is the practical cause of 85% of lung cancers in males whether or not exposed to asbestos (Hammond & Seidman, 1980; Hammond & Selikoff, 1973). In contrast, asbestos would be the predominant cause of mesothelioma in exposed shipyard workers. Although oestrogens are believed to be promoters, they can be regarded as the practical cause of a significant proportion of endometrial cancers in parts of the United States (Gusberg, 1980). Thus, in considering aetiology, it is important not to confuse multifactorial mechanisms, i.e. relative role of initiator versus promoter in cigarettes, individual susceptibility, etc., with practical causes especially since the latter may have more immediate health relevance. Routine epidemiology and long-term experimental testing essentially demonstrate the overall impact of numerous events, and usually the effect of individual modulating factors cannot be estimated.

Role of epidemiology in evaluating aetiological hypotheses

While defined aetiological hypotheses in human cancer have been largely developed through case-history epidemiological studies, it is less widely appreciated that epidemiology may also contribute to the analysis of possible mechanisms for those environmental cancers of uncertain aetiology, through deductions made from geographical and temporal variations in incidence and associated migrant studies (Clemmesen, 1950; Doll, 1967; Higginson, 1969; Higginson & Muir, 1979; Higginson & Oettlé, 1960; Wynder & Gori, 1977). In this context the study of low-risk populations is particularly important in allowing the evaluation of those environmental factors especially related to lifestyle (Enstrom, 1980; Higginson & Oettlé, 1960; Lyon, Gardner & West, 1980; Phillips, Kuzma & Lotz, 1980). Epidemiology is of course complemented by laboratory studies which may not only suggest new aetiological hypotheses for testing in man but also the nature of the mechanisms involved.

It should be emphasized that no epidemiology nor laboratory study, whether relating to discrete carcinogens or lifestyle factors, can now be completely conclusive since all results (positive or negative) are dependent on statistical probabilities (IARC Working Group, 1979a; Vesell, 1980).

Hypotheses on the causation of human cancer

Prior to 1950 the cause of very few human cancers had been firmly established, but considerable data are now available. Most cancers can be described as tumours of well-defined environmental origin, or those for which the environmental background can only be deduced. For a smaller group no satisfactory causal hypotheses are available. Estimates of the proportion of cancers in each aetiological group in different communities are reported elsewhere (Higginson, & Muir, 1979).

Cancers caused by defined exogenous factors

The majority of tumours in this group are epithelial cancers of the skin, respiratory and upper digestive systems, liver and bladder. In addition, the causes of a small number of cancers of the endocrine-dependent organs, haemopoietic system, bone and soft tissues have also been identified.

Personal habits are by far the most important stimuli established, especially cigarette smoking. Studies in all countries emphasize the overwhelming role of this habit which causes between 25–35% of all cancers in males in North America, Europe and Japan (Hammond & Seidman, 1980). The proportion in females is small but increasing rapidly. Cigarette smoking is not only carcinogenic *per se* but also enhances multiplicatively the effects of such other factors as asbestos and alcohol (Hammond & Selikoff, 1973; Tuyns, 1978). In most countries this habit is predominantly responsible for the increases in cancer incidence reported since 1950. Excess alcohol consumption, sunbathing, and in Asia betel-quid chewing are other carcinogenic habits. A much smaller part of the cancer burden even in industrialized countries is related to occupational (probably less than 5%) and iatrogenic and radiation exposures. It is estimated that approximately 39 of the 537 chemicals and related industrial processes evaluated in the IARC Monographs Series are probable or definite human carcinogens, of which the most important of industrial origin is asbestos, especially in association with cigarette smoking. However, the list is not definitive and further iatrogenic and occupational hazards will probably be detected in the future.

Although less well-documented, there is considerable evidence in Africa and Asia that primary liver cancer arises in hepatitis B virus carriers who are exposed to aflatoxin (Larouzé, Blumberg, London, Lustbader, Sankalé & Payet, 1977; Linsell & Peers, 1977).

Cancers of probable environmental origin

This group comprises tumours of the gastro-intestinal tract, e.g. stomach and large intestine, and of the endocrine-dependent organs, e.g. breast, body and cervix of the uterus, and ovary, etc. Although the definitive stimuli have not been identified, the most

rational interpretation of the available epidemiological data would indicate a direct or indirect association with environmental factors (Higginson & Muir, 1976). While it is probable that further strong exogenous agents will eventually be identified for other tumours, e.g. oesophagus in parts of China, Africa and Iran, where precancerous mucosal lesions are found in most adults (Crespi, Muñoz, Grassi, Aramesh, Amiri, Mojtabai & Casale, 1979), this would appear less likely for most cancers in this group and alternative aetiological explanations must be sought.

On one hand, it has been suggested that such cancers largely reflect additive exposures to multiple mutagens or carcinogens within the general environment predominantly of industrial origin (Epstein, 1978). Others believe that most of these cancers are predominantly related to exogenous and/or endogenous factors inherent in life-style modulating cells already initiated by undetermined stimuli, and are not dependent on exogenous initiators alone (Higginson & Oettlé, 1960; Wynder & Gori, 1977), as suggested by much recent experimental work (Slaga, 1980).

The role of ambient environmental pollution

The effects of ambient environmental pollution on several diseases, especially respiratory, are recognized. The fact that exposure to high doses of certain chemicals causes cancer in man and the synergism existing between several toxic agents have led to concern about the significance of multiple low exposures to chemicals in the general environment. Chemicals of natural and synthetic origin with biological activity have been and are ubiquitous in the environment in all countries. They include carcinogens and mutagens, promoters, enhancers, etc. Polycyclic aromatic hydrocarbons, nitrosamines, mycotoxins, flavonoids and other suspected carcinogens have been demonstrated in air, water (Kraybill, 1978; Wilkins, Reiches & Kruse, 1979), food, and alcoholic beverages (Rose, 1977). Many individuals have been and are exposed to many of the animal carcinogens reviewed in the IARC Monographs Series (Supplement 1; IARC Working Group 1979a). The problem of their evaluation at very low levels of exposure is illustrated by the nitrosamines which are not only widely distributed in the environment, but may be demonstrated in body tissues and fluids (Walker, Castegnaro, Griciute & Lyle, 1978; Yamamoto, Yamada & Tanimura, 1980). While many are carcinogenic to several animal species, to date there is no firm evidence of carcinogenic activity in man (Tannenbaum & Young, 1980).

Attempts to evaluate the specific carcinogenic potential to man of an individual chemical at low dose among the myriad chemicals present in the environment poses almost insoluble logistic and technical problems for the epidemiologist, especially in the presence of powerful confounding variables such as cigarettes. The difficulties are even greater than those of determining the effects of low doses of ionizing radiation, a recognized human and animal carcinogen where controversy still exists (Land, 1980), although in this case the "target cell" dose can be estimated.

The value of animal experiments in identifying potential human carcinogens is well recognized, but there is a tendency to concentrate on the statistical limitations of epidemiological methods rather than the equally great limitations of biological extrapolation between species. Such difficulties cannot be overcome simply by theoretical mathematical models extrapolating from animals since many of the pertinent parameters relating to carcinogen metabolism, inhibition, etc., cannot be measured (Coulston, 1979), nor can the dose at the target cell, although newer technology, i.e. measurement of DNA adducts, may permit this possibility in the future (Rajewsky, 1979).

Within limits, however, epidemiology, by comparing cancer patterns in different environments, may help *to evaluate the additional impact of the overall burden of chemicals in a specific environment, i.e. the sum of total increased risks.* Such an approach has been attempted (Higginson, 1979; Lyon *et al.* 1980; Royal Society Study Group, 1978; Wynder & Gori, 1977) and further details may be obtained from these reports. In brief, no consistent relationships have been observed between total cancer patterns or between organ sites, and indices of probable ambient environmental pollution such as industrialization and urbanization. The most intensive studies have been in relation to air pollution, where no significant effect can be demonstrated if correction is made for such variables as cigarette smoking and occupational exposures (Cederlöf, Doll, Fowler, Friberg, Nelson & Vouk, 1978; Goldsmith, 1980; Haenszel, Loveland & Sirken, 1962; Haenszel & Taeuber, 1964; Hammond & Garfinkel, 1980). Such studies do , however, permit partial evaluation of the additive effects of occupational exposures in both urban and non-urban environments (Hammond & Garfinkel, 1980). The above observations are supported in a recent report indicating no significant differences in cancer incidence between urban and rural Mormons (Lyon *et al.* 1980). Thus it appears that only a very small part of the total cancer burden can be directly related to industrialization in a general sense, and alternative aetiological explanations must be considered for the majority of tumours of environmental origin. These observations also have obvious relevance to evaluating the existence of 'no-effect' exposure levels for identified carcinogens or reversibility in carcinogenesis.

Lifestyle

In addition to such clearly defined habits as cigarette smoking, alcohol ingestion, sunbathing and occupation, this term covers the total cultural, behavioural and dietary environment i.e. all exogenous factors inherent in daily life. Lifestyle includes such carcinogenic risk factors as behavioural patterns, e.g. age at first marriage and pregnancy; such physiological parameters as age at menarche or menopause; and such dietary patterns as quantity and quality of dietary fat, fibre etc. While the recognition of 'lifestyle' factors in cancer is not recent (Clemmesen, 1950; Higginson & Oettlé, 1960; Willis, 1948; Wynder & Gori, 1977), it has been neglected due to its poorly defined nature.

Diet should be considered as a highly complex chemical mixture. It not only includes preformed carcinogens and carcinogen precursors but also enhancing and inhibiting factors (Wattenberg, 1979; Wynder, Hoffmann, McCoy, Cohen & Reddy, 1978). Further, while diet might be directly related to stomach or

large intestine cancer through exogenous carcinogens or endogenous carcinogen formation e.g. nitrosamines (Tannenbaum & Young, 1980), it may also affect tumour development of the breast for example in a non-specific manner through variations in fat and calorie intake (Miller, 1980; Newberne & McConnell, 1980; Wynder & Gori, 1977). Further, diet is a determinant in menarche, height, obesity, etc., risk factors for cancers of the breast and endometrium (Hill, Wynder, Barbaczewski, Helman, Hill, Sporangisa & Huskisson, 1980). The nature of such non-specific effects is uncertain but they may operate through the hypophyseal axis. Moreover, numerous studies confirm the role of diet in modulating the induction of enzymes of potential significance in carcinogenesis (CIBA Foundation Symposium 76, 1980; Conney, Pantuck, Pantuck, Buening, Jerina, Fortner, Alvares, Anderson & Kappas, 1978; Kalamegham, Krishnawamy, Krishnamurthy & Bhargava, 1979; Newberne & McConnell, 1980; Vesell, 1980). Calorie and protein reduction have been associated with inhibition of tumours of the breast, intestine and skin in animals and a high-fat diet has been associated with increased cancer in humans and animals possibly due to late-stage effects (Gori, 1978; Hirayama, 1979; Hoehn & Carroll, 1979; Newberne, Weigert & Kula, 1979). The type of carbohydrate has been shown to be important under certain experimental conditions, but in man so far only for fibre is the evidence reasonably strong, notably for cancers of the colon and rectum. While the role of vitamin A deficiency has yet to be demonstrated in man, some studies report that increased vitamin A protects against lung cancer (Wald, Idle, Boreham & Bailey, 1980). Earlier reports that malnutrition was a major factor in liver cancer in Africa have not stood up to further examination (Higginson, 1963). Further, diet also modifies the nature and presence of mutagens in the faeces, some of which have been causally associated with cancer of the large intestine (Dion, Bright-See, Furrer, Eng & Bruce, 1980). Mutagens may arise from normal nutrients during cooking (Kawachi, Nagao, Yahagi, Takahashi, Sugimura, Takayama, Kosuge & Shudo, 1979). Reports relating gastric cancer to nitrate ingestion leading to endogenous formation of nitrosamines remain to be confirmed (Tannenbaum & Young, 1980).

Considerable data have accumulated, indicating that risk factors arising from behaviour and diet may also be associated with biochemical and metabolic variations in the host, especially hormonal variations which have possible relevance to human carcinogenesis (de Waard, 1979; Ernster, Sacks, Selvin & Petrakis, 1979; Gusberg, 1980; Moolgavkar, Day & Stevens, 1980; Ross, Paganini-Hill, Gerkins, Mack, Pfeffer, Arthur & Henderson, 1980; Trichopoulos, Cole, Brown, Goldman & MacMahon, 1980; Vesell, 1980). The promoting role of exogenous oestrogens in endometrial cancer (IARC Working Group, 1979b) and the inhibitory effect of certain contraceptives in endometrial and ovarian cancers also support the possibility that endogenous hormonal factors may be implicated (Cole, 1980; Hulka, Fowler. Kaufman, Grimson, Greenberg, Hogue, Berger & Pulliam, 1980; Smith, Prentice, Thompson & Herrmann, 1975).

Cancer incidence has been shown to be modified by socio-economic gradients (Office of Population Censuses and Surveys, 1978) which are closely associated with diet, behaviour and cultural variations (Morris, 1979). Further, while epidemiological studies on cancer and occupation are classically associated only with the identification of discrete carcinogens, they may be equally informative in evaluating lifestyle and socio-economic factors (Morris, 1979; Office of Population Censuses and Surveys, 1978). Since individual occupations are recruited from specific segments of the community, the health patterns in such occupations reflect the local community environment and socio-economic background. Fox & Adelstein (1978) calculated that most of the differences (88%) in cancer patterns between occupational groups are probably due to lifestyle variations and not to workplace exposures. This is further illustrated by studies on breast and cervical cancers (Devesa & Diamond, 1980; Pell, O'Berg & Karrh, 1978; Trichopoulos, MacMahon & Brown, 1980). Davies, Edmundson, Raffonelli, Cassadi & Morgrade (1972) report that storage of chlorinated hydrocarbons also correlates with socio-economic gradient. Distinction should be made accordingly between tumours due to industrialization *per se* and cancers occurring within a highly developed society.

In contrast to the delay in accepting the role of lifestyle factors in cancer, the concept was accepted more rapidly in the case of cardiovascular disease, and led to considerable publicity on the benefits of dietary and behavioural changes.

Significance of time trends

The process of establishing the relative importance of ambient pollution and lifestyle factors has been complicated by conflicting interpretations of cancer–time trends. The evaluation of such trends is hindered by registration artefacts resulting from variations in population boundaries, census, diagnostic criteria, etc., as well as confounding variables such as cigarette smoking and social habits.

The morbidity data in the US from 1947 to 1970 have been analysed by Devesa & Silverman (1978) who showed that apart from the increase in tobacco- and alcohol-related cancers, other cancers were tending to decrease, trends supported by the mortality data.

Changes since 1970 are especially difficult to interpret (Pollack & Horm, 1980), due to registration artefacts, etc., which have often been ignored. However, the available morbidity and mortality data are in general consistent with earlier observations, and do not suggest real increases in cancers apart from those associated with cultural habits, e.g. tobacco and sunlight. The modest increases in cancers of the breast, prostate and bladder are probably artefactual, e.g. increased histological diagnosis, or changes in lifestyle factors. The latter are also probably responsible for the decrease in gastric and cervical cancer. These interpretations do not imply that localized or general chemical exposures have been completely without effect on general cancer patterns. However, they provide no support for the existence of a new general cancer 'epidemic' apart from the effects of cigarette smoking, alcoholism, and to a much lesser degree asbestos in certain limited population groups.

It has been argued that the increased production of industrial organic chemicals is too recent to permit evaluation of the long-term effects. However, considerable quantities were already in fact produced by 1950 (Davis & Magee, 1979). It should be emphasized that increased production, however, cannot automatically be equated with a comparable increase in carcinogen exposures in man (Lenihan & Fletcher, 1976; Morris, 1975) since public health and industrial controls affecting both ambient and point-source exposures have been implemented in many countries. From the microbiological angle it would be interesting to know if there has been any increase in background mutational rates which could be attributed to variations in the chemical environment over the last 30 yr. The existence of localized areas of unusual cancer incidence, or marked changes in temporal trends requires the continual support of adequate surveillance and monitoring mechanisms.

While attention has been concentrated on changes in chemical exposures, it is frequently forgotten that comparable changes in both dietary and behavioural patterns have occurred in many countries, largely as a result of efforts to control cardiovascular disease, and the effects of these changes on cancer patterns remain to be determined. In the United States, for example, between 1963 and 1977, there was a fall in the *per capita* consumption of tobacco of 29%, of eggs 15%, of milk and cream 23%, of butter 36% and of animal fats and oils 47%, whereas consumption of vegetable fats and oils increased by 58% (McQuade, 1980). Furthermore, between 1910 and 1976, the consumption of carbohydrates fell by 21%, of which the contribution from starches fell by 45% (Brewster & Jacobson, 1978). If such changes are as important in the modification of human cancer patterns as migrant and geographical pathology studies suggest, their effects should be observed during the next few decades, and appropriate action initiated. However, the literature on diet in animal and human cancer indicates many inconsistencies in establishing clear-cut relationships (Higginson & Muir, 1979).

In conclusion, although there is a widespread belief in the importance of dietary factors, and while their role in promotion, enzyme induction, etc., is well-established in experimental animals, their role in man is much less clear. The difficulties of evaluating the significance of modest dietary changes as seen in man in the context of other modulating factors compared with the very marked changes seen in isolated animal experimentation increases the difficulty of extrapolation. This is well illustrated by Hoehn & Carroll (1979) who demonstrated the effects of simple carbohydrate changes on DMBA-induced tumours in rats.

Cancers of unknown aetiology

Only a few discrete causal factors have been determined for most tumours of children, bone and soft tissues and haemopoietic system, the aetiology of the majority being unknown. Most tumours show very modest geographical and temporal variations in incidence, and their relationship to environmental factors cannot accordingly be easily evaluated. It is tempting to regard such cancers as representing a background incidence in man similar to 'spontaneous' tumours in animals which, however, are often related to inherited factors or endogenous viruses.

The implications of epidemiology to the study of carcinogenic mechanisms

The multifactorial nature of human cancer has long been recognized. As the influence of other modulating factors in the early and late stages of carcinogenesis is increasingly recognized (Berenblum, 1979; Higginson, 1980; Miller & Miller, 1979; Wynder *et al.* 1978), it seems less appropriate to consider carcinogenesis in man only in terms of the classical two-stage skin model.

Contrary to recent reports, human cancer has not been considered in terms of single causes by most epidemiologists (Table 1). Kennaway (1950) emphasized the modifying effect of diet. The interplay of several factors was strongly supported by numerous studies such as on alcohol, asbestos and cigarette smoking (Hammond & Selikoff, 1973; Tuyns, 1978; Wynder & Gori, 1977), hepatitis virus in liver cancer (Higginson, 1963), and the interaction of multiple carcinogenic risk factors in cancers of the endocrine-dependent systems (Miller, A. B., 1978). Adenocarcinoma of the vagina due to diethylstilboestrol is also suspected to involve a second factor. Further, the fact that latent carcinoma of the prostate is equally prevalent in most races, whereas the incidence of invasive carcinoma varies widely, strongly suggests an interplay of initiating and promoting factors (Breslow, Chan, Dhom, Drury, Franks, Gellei, Lee, Lundberg, Sparke, Sternby & Tulinius, 1977).

Figures 1 to 3 are diagrammatic representations of the carcinogenic process. While division into early, initiation, and late phases is probably an over-simplification in view of the probable overlaps, the diagram provides a useful base for discussion. It does illustrate moreover that clinical cancer does not represent a single mechanism but a series of events, only a few

Table 1. *Human cancers with evidence of multistage and multifactorial origin*

Site/tumour type	Aetiological factors
Lung	Smoking, asbestos, ionizing radiation
Oesophagus	Alcohol, smoking
Liver	Hepatitis B, mycotoxins
Stomach and large intestine	Endogenous carcinogen formation, non-specific dietary factors
Breast and endometrium	Behaviour, diet, hormonal promotion
Burkitt's lymphoma	EBV, malaria, factor 'X'
Nasopharyngeal carcinoma	Host factor, EBV, factor 'X'

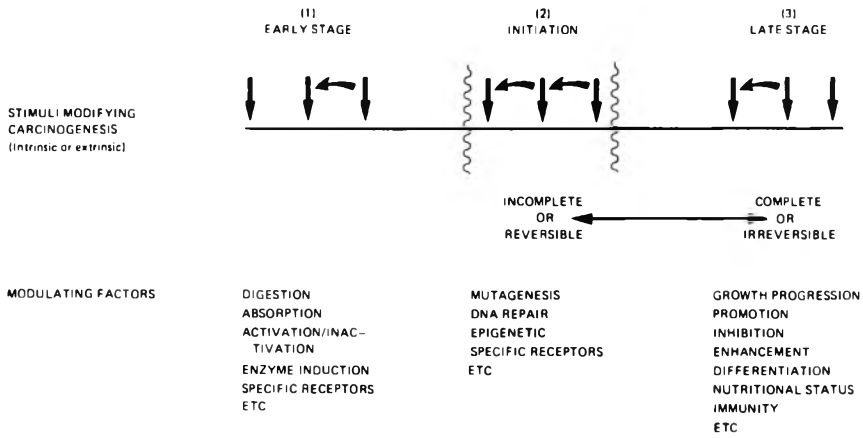

Fig. 1. A simplified diagram showing the hypothetical stages in carcinogenesis and some of the exogenous and endogenous factors possibly involved.

of which have adequately been studied and measured (Vesell, 1980). Although order is essential to the concept of cancer development and progression, there is good experimental evidence that many steps are at least partially reversible (Farber & Cameron, 1980; Singer, 1979).

It seems a reasonable possibility that individual susceptibility and the contribution of lifestyle as indicated by carcinogenic risk factors may eventually be explicable in terms of reversible and irreversible biochemical events and feedback mechanisms operating in both early and late-stage carcinogenesis. Further, there may be both qualitative and quantitative differences between the effects of modulating factors in cases where discrete carcinogens providing a genotoxic stimulus have been identified (Fig. 2), and 'lifestyle' cancers where both extrinsic and intrinsic carcinogenic initiating stimuli may be less important (Fig. 3). The latter illustrates the difficulty of extrapolation from experimental systems, especially for cancers where the modulating factors have yet to be clarified.

The importance of enzyme activation and inactivation of procarcinogens and carcinogens (CIBA Foundation, 1980) is widely recognized. Further, variations in enzyme induction, whether genetically or environmentally determined, may affect both individual susceptibility to carcinogens (Vesell, 1980), and the incidence of certain endocrine-dependent tumours in which subtle variations in hormonal metabolism may be important (Cole, Brown & MacMahon, 1976). Increased extra-glandular formation of oestrogens occurs largely in fat tissues and may explain the relationship between obesity, overeating and endometrial cancer. Endogenous carcinogen formation from carcinogen precursors may be controlled to a considerable degree by environmental factors, by mechanisms including altered enzyme metabolism. Epidemiological studies are only now being developed and will require much more intensive laboratory backup.

Initiating events are easier to explain in terms of genotoxic than non-genotoxic stimuli, since the latter are poorly understood. They may involve gene unmasking, modification of DNA repair, epigenetic changes, etc. (Kroes, 1979), all of which can be environmentally modified and have important implications in terms of the completeness and the reversibility of initiation.

It is thus possible to assume that in man initiation is common but often incomplete or lethal, cancer development being dependent on events in the later stages as well as during initiation (Rajewsky, 1979). This is obviously difficult to demonstrate, but strains of rats with a high frequency of spontaneous hepatomas are regarded as being more susceptible to the promoting action of phenobarbitone than low-incidence strains (Peraino, Staffeldt, Haugen, Lombard, Stevens & Fry, 1980). Similarly, it has been found that initiated but not transformed cells are present in high-skin-cancer strains of mice but not in those of low incidence (S. Yuspa, U. Lichei, D. Morgan & H. Hennings, unpublished data, 1980). In man, the possibility of incomplete initiation is supported by pathological studies on the prostate and breast (Farber & Cameron, 1980), etc. Whether such initiation is due to endogenous agents alone is a matter of speculation. Archer (1980) has suggested that background radi-

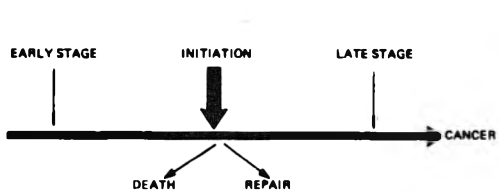

Fig. 2. The predominant effect of a strong discrete initiator (e.g. an alkylating agent) is illustrated in comparison with the slight effects of modulating factors.

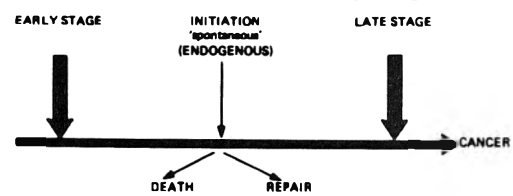

Fig. 3. The relatively important role of 'lifestyle' modulating factors is illustrated in a situation where the role of a direct initiating stimulus may be weak.

ation is a factor of significance in many cancers, but the failure to demonstrate any relation with ambient chemical pollution makes it more difficult to implicate the latter although obviously it cannot be excluded.

The many factors that may be involved in late-stage carcinogenesis have been reviewed (Slaga, 1980). But even for such extensively studied carcinogenic agents in man as cigarettes, the relative importance of initiation and promotion is not understood. However, the fact that the incidence of lung cancer 'freezes' after cessation of smoking suggests that promoting factors are important in later development. In addition to reactions at the target cell, changes in the host may also be pertinent, e.g. immunological factors (Kinlen, Eastwood, Kerr, Moorhead, Oliver, Robinson, de Wardener & Wing, 1980). Conney *et al.* (1978) have demonstrated the role of diet in carcinogen metabolism in man and animals. The slow increases in breast and prostatic cancers in migrants (Haenszel & Kurihara, 1968) show that environmental differences may take several generations to become apparent, and the possibility of sex-linked enzyme imprinting in such cancers in humans should not be ignored. Such a mechanism has been postulated for breast cancers in rats (Mori, Nagasawa & Bern, 1979).

Individual susceptibility

It is not easy to differentiate between environmentally and genetically determined individual susceptibility (Vesell, 1980). Previous attempts to relate lung cancer susceptibility to the induction of aryl hydrocarbon hydroxylase have not been confirmed.

Apart from such clear-cut hereditary syndromes as *xeroderma pigmentosum* and ataxia-telangiectasia, etc., few cancers can be directly attributed to genetic factors. On the other hand, genetic polymorphism may have considerable significance both in relation to enzyme induction and host reactions to exogenous modulating factors (del Villano, Miller, Schacter & Tischfield, 1980; Schull, 1979). Migrant studies, however, emphasize the overwhelming impact of environmental changes.

Conclusions

For nearly 50% of environmentally influenced cancers among men in North America and Europe the main aetiological factors have been identified but the percentage is much lower for women. Many of the causes had already been suggested in the fifties. Since then, much laboratory and epidemiological research has been directed towards investigating the nature of and the mechanisms involved in cancer in man. Increasingly sophisticated technology has been used in attempts to identify the initiation stimuli and the many modulating factors involved. Studies of the progression of cancer and the multi-stage nature of carcinogenesis make it increasingly evident that data from each species are complementary and that an interdisciplinary approach is important. Clearly exposure to high levels of carcinogens must be controlled, but the view that multiple exposure to very low levels of mutagens and carcinogens (general environmental pollution) may have relatively little effect on the total cancer burden indicates the importance of alternative approaches to cancer prevention and control.

The implication of a discrete chemical carcinogen or of a defined cultural habit allows a relatively straightforward approach to cancer prevention. However, the situation has not been so simple in the case of cancers thought to be related to lifestyle, where there have been only very general indications of the factors involved and understanding of the underlying biological mechanisms has been imperfect. It is becoming increasingly clear that combinations of modulating endogenous and exogenous factors may explain the influence of lifestyle based on identifiable biochemical mechanisms. This may eventually allow the possibility of tumour prevention by interference with other stages of carcinogenesis through chemoprevention (Sporn & Newton, 1979), use of cancer inhibitors and dietary changes. The process of attributing many cancers to lifestyle factors has the benefits of directing research attention to factors beyond discrete carcinogens and of arousing intellectual curiosity. Unfortunately, at present it does not provide a basis for the immediate and effective control of many cancers of environmental origin.

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RISK ASSESSMENT AND REGULATORY DECISION MAKING

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Summary—An overview of the regulatory decision-making process is presented, with special reference to the regulation of chemical carcinogens. Current procedures for assessing human risk on the basis of toxicological investigations in animal models are reviewed and a critical appraisal of the available techniques for quantitative risk assessment is provided. The integration of factors other than risk into the regulatory process, including information on health, economic and other benefits, is discussed.

Introduction

Although the nature of the risks confronting mankind has changed dramatically over the years, the concept that risk is an integral component of life has not. Man has always had to contend not only with natural catastrophes and infectious diseases, but also with nutritional deficiencies, toxic moulds, environmental contaminants and extreme climatic conditions. Tannahill (1973) notes that the mean life expectancy of Neanderthal man was twenty years and that less than 10% of the population survived to the ripe old age of forty. Since the beginning of the twentieth century, however, there has been a remarkable improvement in the health status of the Canadian population. Lifespan has been steadily increasing, and many infectious diseases as well as certain degenerative diseases, such as cardiovascular disease and some forms of cancer, are slowly coming under control. These improvements in the overall quality of life may be attributed to a variety of technological developments in the medical sciences and in agricultural and food-marketing practices, although it must be recognized that technological progress is not without its own hazards.

Our transition from a largely rural to a predominantly urban society, coupled with substantial advances in the biological sciences in recent years, has dramatically shifted the public's perception of risk acceptability. Voluntary risks, which our forefathers accepted as an integral part of existence, have become involuntary risks, which the public expects, indeed demands, to have stringently controlled if not eliminated. Much of this public concern stems from a perceived failure of government authorities and industry to deal effectively with risks associated with chemicals in foods, occupational settings and the environment generally.

The use of chemicals in food production and processing has increased dramatically since the turn of the century (Jukes, 1977; Oller, Cairns, Bowman & Fishbein, 1980). Some of these substances are added to food to protect against bacterial deterioration or oxidative changes, while others are used to improve the flavour and texture of food. Pesticides are used to control insects and fungi in agriculture, and certain drugs are used to stimulate growth in food-producing animals. Many of these uses are necessary to sustain the food supply of our ever-burgeoning world population. Thus, it is essential that risk associated with these chemicals be carefully balanced against the health, economic and other benefits that accrue from their use. This is not to say that undue health risks should be tolerated. On the contrary, they should be minimized to the extent technologically feasible, but they must not be eliminated at the cost of compromising the food supply, facilitating the spread of disease, or lowering the overall quality of the environment.

In Canada, as in most nations, the government's intentions with regard to the control of risks are clearly defined. With respect to toxic substances in food, Section 4 of the Canadian Food and Drugs Act (Food and Drug Regulations 1979, Ottawa) provides the Department of Health and Welfare with the legislative power to curtail or eliminate exposure to "poisonous or harmful" substances. A major problem confronting all countries with similar legislation relates to the definition of the terms 'poisonous' and 'harmful'. If we hold the view as toxicologists that these qualities are not inherent vices of a compound but rather a function of dose-dependent toxicity, we have some leeway for permitting the use of chemicals in food. Indeed, it is only by application of such principles that food as we know it can be sold at all. Thus many substances known to produce toxic effects at high doses in laboratory animals are permitted for use in food production or processing. The procedural rules for deciding upon acceptable human exposure levels have been entrenched in regulatory circles for many years, are endorsed by the World Health Organization, and have involved the use of uncertainty factors for translating animal data to man (Vettorazzi, 1980). This procedure is not hard and fixed but is subject to considerable discretion on the part of the regulator, depending upon the nature and degree of hazard involved.

In this paper the role of government in avoiding or reducing exposure to cancer-causing and other harmful agents will be discussed. While problems related to microbiology and nutrition are still very high on our priority list, the pendulum of scientific activity as well

as public concern and debate over food-safety issues has swung over in favour of programmes to detect and eliminate carcinogenic substances. The rapid rate at which we are detecting carcinogens in our environment, coupled with increased consumer awareness, indeed a fear of cancer, has led those of us in government to re-examine critically food-safety policies pertaining to carcinogens as we look towards the future.

Quantitative risk assessment

Safety factors and thresholds

Traditional toxicological procedures define a safe level of exposure for man as some arbitrary fraction of that dose level at which no effects are observed in a group of test animals. For food additives and pesticides inducing toxic effects other than cancer, for example, an acceptable daily intake has often been established through the application of a 100-fold safety factor. This uncertainty factor admits the possibility that man may be up to ten times more sensitive than the animal species tested and allows for a ten-fold variation in sensitivity within the human population (Lehman & Fitzhugh, 1954). The magnitude of the safety factor may be modified depending on the chemical and kinetic properties of the test compound and the effects induced, as well as on the quality of the available toxicological data (Committee on Food Protection, 1980; Safe Drinking Water Committee, 1980).

The use of safety factors in arriving at acceptable human exposure levels would appear to rest at least tacitly on the assumption of the existence of a threshold dose below which no adverse effects will occur. However, it is precisely because the threshold concept may not be universally applicable to carcinogenesis that the regulation of carcinogens is regarded as a unique issue in food safety. This uncertainty as to the low-dose effects of carcinogenic agents has resulted in the proposed use of safety factors as high as 5000-fold (Truhaut, 1979; Weil, 1972).

In mathematical terms, the absence of a threshold precludes the possibility that a sufficiently low level of exposure will be free of any attendant degree of hazard. Biological arguments in favour of the no-threshold concept for carcinogenesis are generally based on the fact that irreversible self-replicating lesions may result from a mutation in a single somatic cell, often following the administration of only a single dose. Arguments against this position draw on the existence of metabolic detoxification, DNA repair, immunological surveillance and other mechanisms that may operate to nullify effects at low doses. Even admitting their existence, thresholds are likely to vary among individuals. The determination of a population threshold thus presents the difficult statistical problem of determining the minimum of the individual thresholds, a minimum which may well be effectively zero in some cases (Brown, 1976).

The safety factor approach has also been criticized on the grounds that the observed no-effect level will depend on the sample size, with response rates of 0/10 and 0/1000 obviously having different interpretations. Moreover, there is always the possibility of observing no effects even though the test compound may affect an appreciable proportion of the population at risk. For example, with fifty animals on test, there is a better than even chance of observing no effects with a compound for which the population risk is actually as high as 1% (Cornfield, Carlborg & Van Ryzin, 1978).

Less generally recognized is the fact that the application of a standard safety factor does not take into account the slope of the dose–response curve for the particular response of interest (Cornfield, Rai & Van Ryzin, 1980). Clearly, a moderate safety factor may provide an adequate margin of safety if the dose–response relationship is relatively steep but may not be sufficiently conservative if the dose–response curve is relatively shallow. Conversely, the universal application of a very large safety factor will result in tolerances that will often be unduly low.

This brings us to the hub of a debate that rages among toxicologists and statisticians alike. This discussion concerns the use of mathematical models to evaluate risks at low doses—doses to which humans may be exposed. The use of point estimates of risk as a major decision criterion in the regulatory control of carcinogens would permit an assessment of the relative risks due to various compounds. Although this perhaps represents the ultimate application of mathematical modelling techniques, knowledge deficiencies in the science base at present preclude the possibility of realizing the full potential of the procedures currently available. However, it is instructive to review briefly where we now stand on the application of procedures for quantitative risk assessment for purposes of regulatory decision making.

Mathematical models and virtual safety

Statistical procedures for quantitative risk assessment involve a mathematical model relating the probability of an induced response to the dose rate. Because of the statistical problems inherent in the determination of no-effect levels, most mathematical models have dispensed with the threshold concept. [Cornfield (1977) has discussed a kinetic model which leads to the existence of thresholds under steady-state conditions. As noted by Brown, Fears, Gail, Schneiderman, Tarone & Mantel (1978), however, the possibility of a response being induced by the reactive metabolite formed during the approach to steady state results in some degree of risk no matter how small the dose.] While absolute safety may be guaranteed in the absence of a threshold only when the level of exposure is zero, a virtually safe level of exposure associated with some suitably low level of risk may still be estimated (Fig. 1). It is important to recognize that since direct estimates of risk at low levels of exposure would require the testing or prohibitively large numbers of animals, the determination of a virtually safe dose will generally involve extrapolation of the experimental results well outside the observable response range.

A number of existing models that have been discussed in the literature are given in Table 1 (Krewski & Brown, 1981). Statistical models are based on the notion that each individual in the population has his own tolerance to the test compound. Any level of exposure below this tolerance will have no effect on the individual, while any level of exposure exceeding the tolerance will result in a positive response. These tolerances are presumed to vary among individuals in

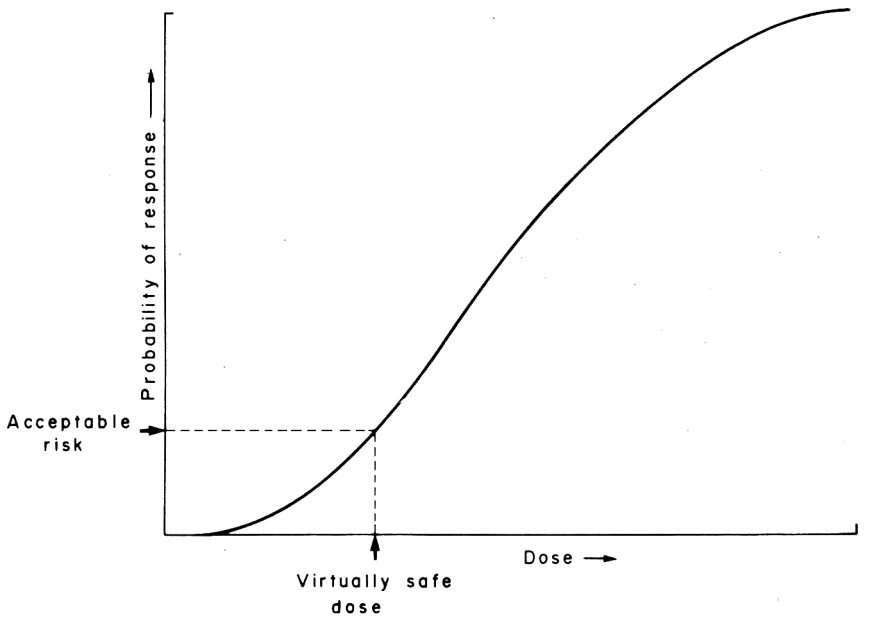

Fig. 1. Determination of a virtually safe dose.

the population, with the lack of a population threshold reflected in the fact that the minimum tolerance is allowed to be zero. Specification of a functional form for the distribution of tolerances determines the shape of the dose–response curve and thus defines a particular statistical model. Although the choice of the tolerance distribution is to a large extent arbitrary, three commonly encountered models of this type are the probit, logit and Weibull. [Certain statistical models may also be formulated in terms of time to response considerations. Chand & Hoel (1974) have shown that the probit model rises when the time to response follows a lognormal distribution, with the median time to response satisfying the Druckrey equation.]

Stochastic models are based on the premise that a positive response is the result of the random occurrence of one or more biological events. The one-hit model (Hoel, Gaylor, Kirschstein, Saffiotti & Schneiderman, 1975) is based on the concept that a response will occur after the target site has been hit by a single biologically effective unit of dose. The multi-hit model (Rai & Van Ryzin, 1981) is a direct extension of the one-hit model, assuming that more than one hit is required in order to induce a response. [This model may also be viewed as a tolerance distribution model, where the tolerance distribution is gamma. This formulation allows the 'hit' parameter k to assume non-integral values.] The multi-stage model, on the other hand, is based on the assumption that the induction of irreversible self-replicating toxic effects such as carcinogenesis is the result of the occurrence of a number of different random biological events, the time rate of

Table 1. *Mathematical models and their low-dose behaviour in the case of zero background*

Model	Probability P(d) of a response at dose d†		Low-dose behaviour‡		
			Linear	Sublinear	Supralinear
Probit	$(2\pi)^{-1/2}\int_{-\infty}^{\alpha+\beta\log d}\exp(-u^2/2)\,du$	$(\beta > 0)$	—	$\beta > 0$	—
Logit	$[1+\exp(-\alpha-\beta\log d)]^{-1}$	$(\beta > 0)$	$\beta = 1$	$\beta > 1$	$\beta < 1$
Weibull	$1-\exp(-\lambda d^m)$	$(\lambda, m > 0)$	$m = 1$	$m > 1$	$m < 1$
One-hit	$1-\exp(-\lambda d)$	$(\lambda > 0)$	$\lambda > 0$	—	—
Multi-hit	$[\Gamma(k)]^{-1}\int_0^{\lambda d}u^{k-1}\exp(-u)\,du$	$(\lambda, k > 0)$	$k = 1$	$k > 1$	$k < 1$
Multi-stage	$1-\exp\left(-\sum_{i=1}^{k}\beta_i d^i\right)$	$(\beta_i \geq 0)$	$\beta_1 > 0$	$\beta_1 = 0$	—

†With independent background, the probability of a response at dose d is given by $P^*(d) = \gamma + (1-\gamma)P(d)$, where γ $(0 < \gamma < 1)$ denotes the spontaneous response rate. Under additive background, $P^*(d) = P(d+\delta)$ where $\delta > 0$ denotes the effective 'background' dose.

‡Low-dose behaviour for independent background also. (All models are linear at low doses under additive background.)

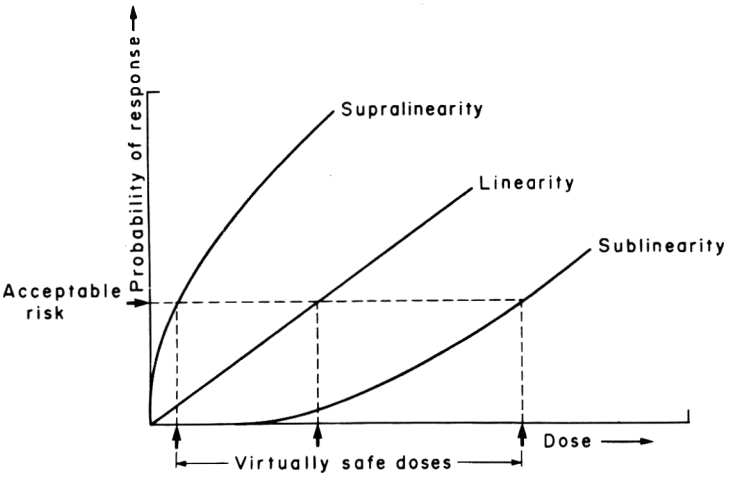

Fig. 2. Linearity, sublinearity and supralinearity at low doses.

occurrence of each event being in strict linear proportion to the dose rate (Crump, 1979; Crump, Hoel, Langley & Peto, 1976). Despite their biological rationale, these stochastic models must also be considered somewhat arbitrary until the mechanisms of carcinogenesis are more fully understood.

The shape of the dose–response curves for the above models in the low-dose region (Table 1) will have considerable impact on estimates of risk associated with low levels of exposure (Fig. 2). The one-hit model is linear at low doses and will thus generally provide relatively high estimates of risk at low dose levels. The logit, Weibull and multi-hit models are linear at low doses only when the shape parameters β, m and k in these models are equal to unity. When these parameters are greater than unity, the dose–response curves for these models approach zero at a slower than linear, or sublinear, rate. The multi-stage model is linear at low doses only when the linear coefficient in the model (β_1) is positive and is sublinear otherwise. The probit model is inherently sublinear at low doses and generally leads to relatively low estimates of risk at low dose levels. [Mathematically, the probit model is extremely flat in the low-dose region, with the dose–response curve approaching zero more rapidly than any power of dose.]

The dose–response curves for the logit, Weibull and multi-hit models can approach zero at a faster than linear, or supralinear, rate, although the biological plausibility of this behaviour seems questionable. From the tolerance distribution point of view, this would indicate that a very high proportion of the population consists of highly susceptible individuals. An example of such behaviour is provided by data on the induction of liver tumours in the rat following inhalation of vinyl chloride (Maltoni, 1975). However, subsequent studies have revealed that the metabolism of vinyl chloride may not be directly proportional to the administered dose (Gehring, Watanabe & Park, 1978). If the concentration in the target tissue is used as the dose metameter for extrapolation, the logit, Weibull or multi-hit models will be very nearly linear at low doses (Van Ryzin & Rai, 1980). This example illustrates the need for supplementary metabolic and pharmacokinetic data in order to determine the appropriate dose metameter for extrapolation (Reitz, Quast, Schumann, Watanabe & Gehring, 1980a; Safe Drinking Water Committee, 1980).

Crump & Masterman (1979) have pointed out that low-dose linearity in the logit, Weibull and multi-hit models is compatible only with dose–response curves that are linear at low and moderate doses and exhibit downward curvature at high doses. Thus, those frequently encountered data sets exhibiting a strong degree of upward curvature at moderate or high dose levels would preclude the existence of low-dose linearity according to these models. The multi-stage model does, however, provide for data that are linear at low doses and exhibit upward curvature at higher doses. (This would be the case when both β_1 and higher-order coefficients are positive.) The one-hit model does not provide for upward curvature in the dose–reponse curve and will not be considered further other than as a special case of the multi-hit, multi-stage and Weibull models with k or m equal to unity.

In the presence of spontaneously occurring responses, a virtually safe level of exposure may be defined in terms of the acceptable increment in the added risk over background (Fig. 3). This background may be assumed to be independent of the induced responses or additive in a mechanistic manner (Hoel, 1980). Assuming independence, the low-dose behaviour of the added risk may be nonlinear as in the case of no background. Assuming additivity, however, the added risk will quite generally be linear at low doses (Crump *et al.* 1976)*. This conclusion is valid in those cases where the test compound increases the spontaneous rate of response through the acceleration of an already ongoing process. The potential for synergistic effects takes on special significance here, in view of the possible dose-wise additivity of the effects of the test compound and those of carcinogenic compounds already present in the environment. While this argument rests solidly on the assumption of additivity, the

*In the kinetic model considered by Cornfield (1977), a reversible metabolic detoxification mechanism also implies low-dose linearity.

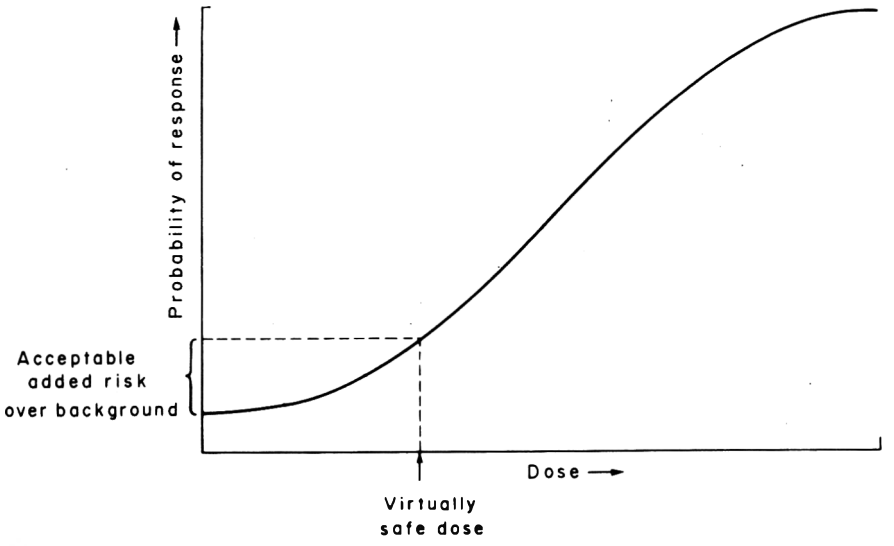

Fig. 3. Determination of a virtually safe dose in the presence of background.

extent to which the assumption of independence or additivity is indicated by either biological theory or experimental data is somewhat unclear at this time (Food Safety Council, 1980a).

Peto (1978) has suggested that upper confidence limits on risk should be linear at low doses, arguing that if the true dose–response curve *could* be linear at low doses, then upper confidence limits on risk *must* be linear at low doses. Confidence limits based on the multi-stage model will possess this property because even if the linear term in the model is estimated to be zero, its upper confidence limit will be greater than zero (Mantel, 1977). In general, upper confidence limits on risk based on other models that may be sublinear in the low-dose region will not possess this property.

A simple extrapolation procedure (Gaylor & Kodell, 1980; Van Ryzin, 1980), which provides for the possibility of low-dose linearity, involves fitting a suitable model to the experimental data and then extrapolating linearly from some point on the fitted curve where the excess risk is still within the observable range (Fig. 4). This procedure not only accommodates low-dose linearity, but will provide a conservative upper limit on risk at low levels of exposure whenever the true dose–response curve is sublinear in the low-dose region. An alternative linear extrapolation procedure which exploits the linearity property of the upper confidence limits on risk based on the multi-stage model is discussed by Crump (1981a).

Evaluation of mathematical models

Empirical results—

Estimates of the added risk over background at low dose levels may be obtained by fitting a particular dose–response model to experimental results obtained within the observable range and then extrapolating to the low-dose region of interest. This procedure will be illustrated using the bioassay data (Table 2) on

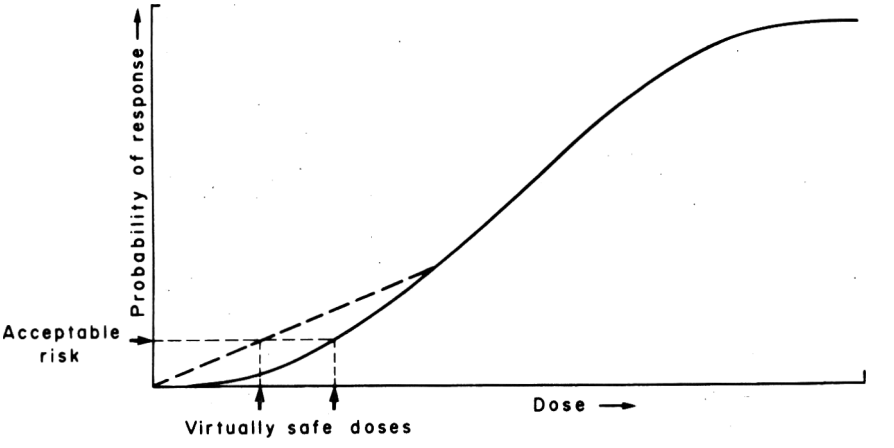

Fig. 4. Linear extrapolation with a sublinear dose–response curve.

Table 2. *Carcinogenic responses induced by four compounds*

Substance	Species	Dose units	Period of exposure (months)	Response
NTA	Rat	% in diet	24	Kidney tumours
Saccharin	Rat	% in diet	24	Bladder tumours
2-AAF	Mouse	ppm in diet	18	Liver tumours
Aflatoxin	Rat	ppb in diet	24	Liver tumours

NTA = Nitrilotriacetic acid 2-AAF = 2-Acetylaminofluorene

nitrilotriacetic acid (NTA), sodium saccharin, 2-acetylaminofluorene (2-AAF) and aflatoxin considered by Krewski & Van Ryzin (1981). The actual data for these four compounds are displayed in graphical form in Fig. 5. along with the fitted Weibull model. The dose–response curves for saccharin and NTA are very steep, while that for 2-AAF is more or less linear throughout the entire dose range. The dose–response curve for aflatoxin is neither as steep as those for NTA and saccharin nor as shallow as that for 2-AAF.

Point estimates of the added risk over background* at low doses obtained using the probit, logit, Weibull, multi-hit and multi-stage models with independent background are shown in Fig. 5, along with the results of linear extrapolation (from the 1% response rate based on the fitted multi-stage model). The linearity of these log–log plots is to be expected at low doses for all but the probit model and provides a convenient means of summarizing the predicted risks on the basis of the different extrapolation procedures.

These results clearly indicate that there are substantial differences among the six procedures considered. Linear extrapolation leads to the most conservative results in all cases, followed by the multi-stage and Weibull models respectively. (Note that the results based on the multi-stage model are, however, close to those based on linear extrapolation in the three cases where the estimate of the linear term in the model is positive.) Estimates based on the logit and multi-hit models are in good agreement, but are less conservative than those based on the Weibull model. The least conservative results are provided by the probit model.

Simulation results—

In order to evaluate further the utility of mathematical models for risk assessment, we will consider two additional examples (Fig. 6). In both examples, the hypothetical dose–response curves exhibit the strong upward curvature observed previously with saccharin and NTA. While the two dose–response curves are similar in appearance, the first curve is linear in the low-dose region while the second is sublinear. Dose–response curves of the former type could conceivably arise when the response of interest may be induced *via* two different mechanisms, with the two corresponding dose–response curves being linear and sublinear, respectively, at low doses. [If the dose–response curves corresponding to these two mechanisms were given by the one-hit model $P_1(d) = 1 - \exp(-\lambda_1 d)$ and the Weibull model $P_2(d) = 1 - \exp(-\lambda_2 d^m)$ with $m > 1$, then the overall dose–response curve would be given by $P(d) = 1 - \exp(-\lambda_1 d - \lambda_2 d^m)$, assuming that the two mechanisms acted independently.] This could be the case, for example, with compounds inducing tumours both directly *via* alkylation of DNA and indirectly *via* faulty DNA synthesis during

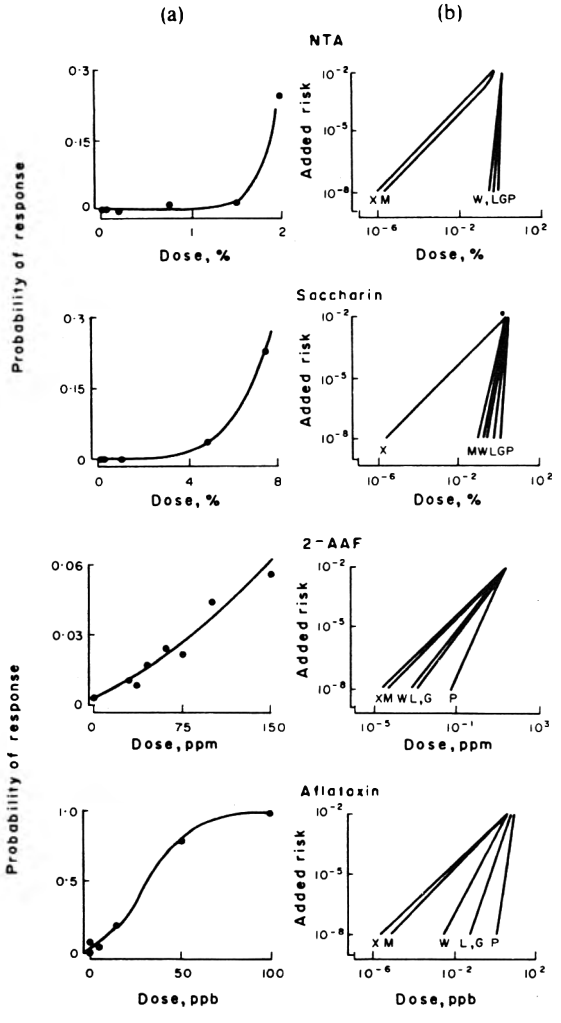

Fig. 5. (a) Weibull dose–response models fitted to the observed data for four compounds (nitrilotriacetic acid (NTA), sodium saccharin, 2-acetylaminofluorene (2-AAF) and aflatoxin) and (b) estimates of the added risk over background based on six extrapolation procedures: X—linear extrapolation; M—multi-stage model; W—Weibull model; L—logit model; G—multi-hit model; P—probit model.

*Confidence limits on the added risk over background are discussed in detail by Krewski & Van Ryzin (1981).

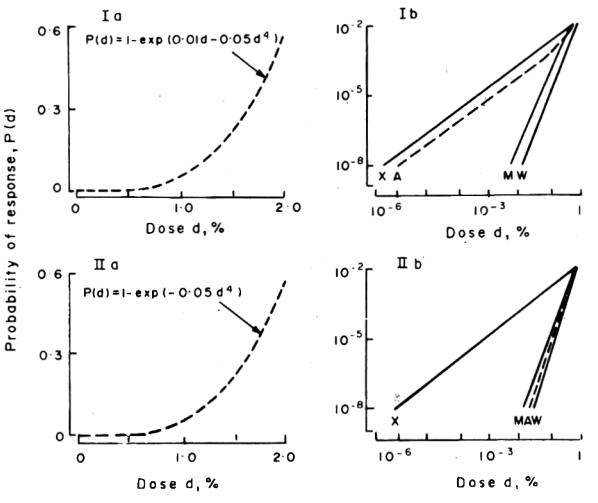

Fig. 6. Hypothetical dose–response curves (a), exhibiting (Ia) low-dose linearity and (IIa) low-dose sublinearity, together with corresponding estimates of added risk over background (Ib and IIb), based on three extrapolation procedures: X—linear extrapolation; M—multi-stage model; W—Weibull model; A—actual risk (estimates averaged over 250 simulated experimental outcomes).

cell regeneration resulting from cytotoxicity (Reitz, Watanabe, McKenna, Quast & Gehring, 1980b; Watanabe, Reitz, Schumann, McKenna, Quast & Gehring, 1979).

The two hypothetical dose–response curves may be used to investigate the low-dose behaviour of the different procedures for quantitative risk assessment through computer simulation. The advantage of this approach is that the actual risk at low doses is known and provides a yardstick against which to measure the performance of the different extrapolation procedures. In this regard, 250 experimental outcomes* were simulated in accordance with the response probabilities given by the assumed models. Estimates of risk in the low-dose region were calculated for each such outcome using the multi-stage and Weibull models as well as the linear extrapolation procedure discussed previously and were then averaged over the 250 'experiments'.

On the average, both the multi-stage and Weibull models underestimate the actual risk in the first example, whereas linear extrapolation leads to estimates of risk close to the true risk (Fig. 6). The corresponding estimates of risk in the second example are similar, even though the actual risk at low doses is much lower because of the sublinearity in the underlying dose–response curve.

In the case of the multi-stage model, it is worth noting that in both examples, averaging over only those experimental outcomes where the linear term in the model is greater than zero leads to estimates of risk close to those based on linear extrapolation, whereas averaging over the remaining outcomes leads to results close to those shown for the Weibull model. This reflects the instability noted earlier in the analysis of the results for saccharin and NTA. This instability can be eliminated through the use of statistical confidence limits, although at the price of imposing low-dose linearity.

*The experimental design used involved four dose levels (0.5, 1.0, 1.5 and 2.0% in the diet) with 100 animals at each dose.

Current status of quantitative risk assessment

The empirical results presented above serve to focus attention on the marked differences in the estimates of risk at low doses based on different mathematical models. Because all of these models fit the data more or less equally well in the observable range, it is difficult to select an appropriate model or range of risks using statistical goodness-of-fit criteria alone. Recent theoretical results by Crump (1981b) in fact indicate that statistical discrimination between two plausible models is difficult even with an experiment designed specifically for this purpose.

The simulation results further demonstrate that models such as the multi-stage and Weibull, which admit the possibility of sublinearity in the low-dose region, can seriously underestimate the true risk when the dose–response relationship is nearly linear at very low doses but exhibits strong upward curvature at higher doses. Conversely, when the linear component of the dose–response curve is absent, linear extrapolation can be excessively conservative. Again, resolution of the question as to whether or not the dose–response curve is linear at low doses is difficult on statistical grounds alone (Crump, Guess & Deal, 1977).

Because of the uncertainties involved in assessing risks of low levels of exposure, some regulatory authorities have advocated the use of conservative risk-assessment procedures (Interagency Regulatory Liaison Group, 1979; US Environmental Protection Agency, 1980). While linear extrapolation may be appropriate for potent electrophilic carcinogens, the use of such conservative procedures for less potent substances, which may induce tumours through per-

turbation of normal physiology, may not be warranted. In the latter case, however, the most suitable model for extrapolation is not at all clear. In general, estimates of risk based on the probit model may be over liberal because of extreme steepness in the low-dose region (Hartley & Sielken, 1977). This may also be true to a lesser degree with the multi-hit and logit models (Haseman, Hoel & Jennrich, 1981), both of which are generally in close agreement. The Weibull and multi-stage models are more conservative, although the latter model can lead to estimates as conservative as those based on linear extrapolation when the linear term in the model is estimated to be greater than zero.

The problems of low-dose extrapolation are further compounded by the uncertainties involved in the extrapolation of cancer data from animals to man. One cannot be certain that the target tissue will be the same in different species (Campbell, 1980; Tomatis, Agthe, Bartsch, Huff, Montesano, Saracci, Walker & Wilbourn, 1978), let alone the potency of the test compound or the shape of the dose–response curve. Even among different strains of the same species, dose–response relationships can vary markedly (Haseman & Hoel, 1979). Determination of the levels of human exposure to food chemicals, and hence the corresponding risk, requires accurate information on food-consumption patterns as well as on the concentration of these chemicals in the food supply (Food Safety Council, 1978).

This inability to assess, with a reasonable degree of certainty, the risk to man at low levels of exposure continues to present serious problems in regulatory applications. For example, the National Academy of Sciences (1978), in its report on saccharin, estimated that the expected number of cases of bladder cancer in the United States due to exposure to 120 mg saccharin/day may range from as low as 0·22 over the next 70 yr to as high as 1,144,000. These estimates of risk span a range of eight orders of magnitude and are of rather limited assistance to regulators charged with the responsibility of making critical public health decisions.

Future perspectives

While mathematical models may take greater cognizance of the shape of the dose–response curve and the sample size than do safety factors, the selection of an appropriate model for extrapolation in cases where the assumption of low-dose linearity is considered unwarranted is difficult. Nonetheless, this procedure still holds promise of providing a more orderly and systematic approach to tolerance determination than the application of an arbitrary safety factor. Further research into the performance of different models within a variety of chemical classes determined by the physical, biological and biochemical properties of the test compound may assist in the resolution of this problem in the future. Ultimately, a deeper understanding of the mechanisms of carcinogenesis and other toxic phenomena should provide a firmer basis for the selection or development of appropriate models for extrapolation.

In the meantime, systematic procedures need to be established to rank our level of concern over cancer-producing substances of varying potency (National Academy of Sciences, 1979). [The classification scheme discussed by Greisemer & Cueto (1980) refers to the degree of experimental evidence for carcinogenicity (i.e. whether or not effects are observed in multiple species or strains, multiple experiments or to an unusual degree) rather than carcinogenic activity or potency.] Experience in experimental carcinogenesis to date suggests that many of the more potent animal and human carcinogens share a number of common characteristics in terms of their biological activity. For example, it is now well established that carcinogens such as aflatoxin and 2-AAF are metabolized to proximate electrophilic compounds which alkylate DNA bases (Miller & Miller, 1976). Such substances are usually carcinogenic in more than one species and produce tumours at multiple sites, at least at high doses. In most instances, the latency period is relatively short and is inversely related to dose level. These substances usually produce a significant proportion of malignant tumours as well as a spectrum of preneoplastic and benign lesions; most of them also produce other forms of toxicity including progressive pathological changes in target organs. Other properties shared by these chemicals include their cytotoxicity in cell culture and activity in short-term *in vitro* tests for carcinogenicity and genotoxicity. In this connection, many of the well-known electrophilic carcinogens produce positive results in the Ames test (McCann, Choi, Yamasaki & Ames, 1975) and are active in transforming cells *in vitro* (Pienta, 1980).

On the other end of the spectrum there are substances, such as NTA and saccharin, that induce tumours in animals but only at high doses or under special test conditions. For example, NTA shows a steep dose–response curve for kidney-tumour induction. It is not mutagenic in Drosophila (Kramers, 1976), and additional studies strongly suggest that it is negative in other well-validated short-term tests (National Toxicology Program, 1979). Saccharin is somewhat similar in terms of its steep dose–response curve for tumour induction and its weak potential for genotoxicity (Ashby, Styles, Anderson & Paton, 1978). It is also known to demonstrate tumour-enhancing or promoting action following the administration of known bladder carcinogens (Cohen, Arai, Jacobs & Friedell, 1979; Nobanski, Hagiwara, Shibata, Imalda, Tatematsu & Ito, 1980).

Clearly, many intermediate categories may be constructed between these two extremes, using the following criteria as a guide (Munroe, 1980; Squire, 1981):

Number of species and strains affected;
Number of tissue sites at which tumours occur;
Latency period;
Strength of dose required to induce tumours;
Proportion of benign and malignant lesions;
Nature and degree of other pathological changes;
Chemical similarity to other known carcinogens;
Metabolic and pharmacokinetic data;
Biochemical reactivity with DNA, RNA and protein;
Genotoxicity and activity in short-term tests for carcinogenicity.

The difficulties in applying a more elaborate classification scheme, however, are easily envisaged.

Certain carcinogens, such as those that act by pro-

ducing a hormonal imbalance (Truhaut, 1980), will no doubt be difficult to classify. As our knowledge of the mechanisms of carcinogenesis expands, however, it will become possible to refine the simple dichotomous classification discussed above, especially for the evaluation of non-genotoxic carcinogens and tumour promoters. Nonetheless, it is clear that we should be more concerned with the more potent compounds that demonstrate classical carcinogenic activity than with those that appear to act by overwhelming biochemical and physiological mechanisms and produce tumours only at near-toxic doses (Shank & Barrows, 1981).

Regulatory decision making

While it is a worthwhile and, indeed, important endeavour to improve our ability to assess risk, this scientific issue must be placed in the proper context. It is becoming increasingly apparent that responsible regulatory decisions regarding the fate of toxic environmental chemicals require a careful evaluation of a host of health, economic and social factors in addition to a thorough evaluation of potential risks. The balancing of health and other benefits against the perceived risk has thus become a central issue in the development of regulatory policy (Calkins, Dixon, Gerber, Zarin & Omenn, 1980; Gori, 1980). Because the uncertainties involved in assessing benefits are even greater than those involved in assessing risk (Darby, 1980; Miller, 1979), the incorporation of factors other than risk into the decision-making process is generally discussed in terms of general guidelines to be applied on a case-by-case basis (Food Safety Council, 1980b). Regardless of the extent to which information on benefits may be quantified, it is clear that a responsible and equitable regulatory system should, in addition to establishing levels of categories of risk, provide for a weighing of health and other benefits against risk, thereby generating a variety of viable regulatory options for consideration (Miller, 1980).

This process may be systematized to the extent that the following factors are balanced against the perceived risk:

Consumer expectations;
Education to permit informed choice by consumers;
Cost to industry and ultimately to consumers;
Ability to control exposure—consumption monitoring programmes;
Impact on trade;
Availability of less hazardous substitutes;
Ability to enforce regulations;
Impact on future regulatory policy.

These factors are purposely not ranked in order of priority, since each case is necessarily evaluated individually using these criteria as guiding principles. Thus, it is likely that the relative weight assigned to each factor will shift from one situation to another.

In Canada, we are guided to a considerable degree by consumer expectations regarding food safety, reflected, in part, through elected parliamentary representatives. As far as is possible, regulatory decisions take serious consideration of consumer wishes and at present formal mechanisms are being established to permit broader consumer input into the decision-making process. The views of consumers are also heard through inclusion of some of the consumer associations in *ad hoc* advisory panels of medical and nutritional experts, and of course their views have a considerable impact on final decisions.

For the most part, consumers expect their food to be free from residues of chemical substances. In practical terms this is an unachievable goal. Thus, it is incumbent upon regulatory agencies to inform consumers through labelling and other informational programmes genuinely designed to permit informed judgements to be made regarding risks associated with food products. Perhaps there is no greater challenge facing regulatory agencies in the coming years than the question of the growing credibility gap in the public's mind regarding regulatory policy on carcinogens (Handler, 1980).

The impact of regulatory decisions on costs to industry—costs that may be passed on to consumers—is an important consideration. In Canada we are currently committed to a programme of de-regulation where desirable, and every effort is being made to ensure that unnecessary regulations are revoked. Excessive and sometimes unnecessary regulation of industry ultimately leads to increased food costs and, given the present inflationary trend, the cost of food is an important component of safety decision making. A recent Canadian government policy directive requires that a detailed socio-economic impact statement be prepared for any decision related to health, fraud or safety if it is expected to have an annual economic impact on industry in excess of ten million dollars (Treasury Board of Canada, 1979).

It is becoming increasingly clear that risk assessment requires a critical evaluation of intake patterns of food chemicals by consumers. We are moving ahead in the development of a food-consumption monitoring programme. It is envisaged that this programme will provide more accurate information regarding the intake of food ingredients than is now available and will permit an assessment of intake in relation to established tolerable intakes.

When an undue health risk associated with the use of a particular compound is identified, consideration may be given to the use of less hazardous substitutes. This was the case when preliminary findings (Comptroller General of the United States, 1980) recently suggested that sodium nitrite might induce tumours of the lymphatic system in test animals*. Because of its widespread use as a preservative and colour and flavour additive in processed meat, fish, poultry and other products, and as an inhibitor of the growth of the bacterium producing the deadly botulinum toxin, nitrite offers significant convenience, appeal and health benefits. Alternative means of preventing botulism were given consideration at the time and included refrigeration and freezing, thermal processing, irradiation, freeze-drying and chemical substitutes such as potassium sorbate. In evaluating such alterna-

*A subsequent review of these results failed to confirm the preliminary findings (Interagency Working Group on Nitrite Research, 1980).

tives, due consideration must be given to their efficacy, technological feasibility on a commercial scale and cost, as well as to the impact on the existing food supply during the implementation period.

The impact of regulatory decisions on the international food trade must also be given serious consideration. The potential for establishing non-tariff trade barriers is easily recognized if one reflects for a moment on the fact that allura red or FD & C Red No. 40 is permitted in the USA but not in Canada, while the reverse is true with amaranth or FD & C Red No. 2 (Lagakos & Mosteller, 1981). In keeping with our international obligations we are committed through the Codex Alimentarius Commission to harmonize food standards where possible, thus purposely eliminating non-tariff barriers. We are therefore strongly supportive of the World Health Organization's activity in relation to safety evaluation of chemicals, particularly the activities of the Joint Expert Committee on Food Additives and the Joint Expert Committee on Pesticides. It is through these channels that we envisage the establishment of an international forum for risk assessment in keeping with the World Health Organization's aims expressed through the new International Programme on Chemical Safety (Vouk, 1980).

Regulations must have a meaningful purpose and it is counterproductive to introduce regulations that cannot be enforced. A decision to promulgate regulations must be accompanied by a careful evaluation of resource implications for enforcement measured against effectiveness in terms of increased consumer protection. This is a difficult task, but in these times of restraint on government spending it surely ranks as an important component of the decision-making process.

Finally, it is important to discuss briefly the impact of decisions on future policy considerations. In this respect the establishment of precedent-setting steps in food-safety policy must include a thoughtful analysis as to how that decision will influence possible future decisions. We must try to predict the future course of events as well as assess the relative importance that may be assigned to any of the previous considerations in the future. We also must never lose sight of the fact that we live in a democracy and that guidance on questions of policy has its roots in consumer wishes funnelled to us through elected representatives.

Conclusions

In this paper, we have critically examined the status of risk assessment in the regulatory process. This analysis indicates that we must not delude ourselves into thinking that the current state of the art of quantitative risk assessment will permit its unqualified application in regulatory decision making. The quantification of human risk on the basis of the results of laboratory studies in animals should be approached with great caution. We must not lose sight of the fact that animal studies serve primarily as a qualitative surrogate for humans and that any attempts to quantify responses beyond the realm of biological certainty are open to serious question. Thus, we are obliged to make clear to regulators, industrialists and the public the real magnitude of the uncertainty that surrounds the risk assessment process. To do otherwise would create a false impression of the realities of safety evaluation as it is now practised.

Looking to the future, we shall no doubt attain a deeper understanding of molecular events in toxicology, permitting us to put greater reliance on quantitative risk estimation through the development of more realistic and reliable methods for extrapolation. Likewise, epidemiological procedures including post-market surveillance of new chemicals introduced to the market place must surely be considered important tools for the identification and quantification of human risk.

Increasingly, regulators will be required to consider factors other than risk in the decision analysis process. Thus, health, economic, convenience and appeal benefits will play a larger role in an expanding regulatory arena. In addition, regulators will be required to examine critically the impact of decisions on international trade agreements, as well as to assess the cost-effectiveness of regulation in terms of a net gain in public health.

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THE DECISION-POINT APPROACH FOR SYSTEMATIC CARCINOGEN TESTING*

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Summary—Advances in the understanding of the mechanisms of chemical carcinogenesis suggest new approaches to the practical aspects of the bioassay of carcinogens and regulatory impact, and to the determination of health risk. Chemical carcinogens have been classified into two main types on the basis of their specific properties: (1) genotoxic carcinogens; (2) agents operating by epigenetic mechanisms. Current concepts indicate that genotoxic carcinogens require distinct qualitative and quantitative types of analysis since their fundamental mechanisms of operation are different from those of epigenetic agents. A systematic 'decision-point approach' to carcinogen testing provides for distinction between genotoxic and epigenetic carcinogens. The first set of data points involves the following: (1) structure–activity relationships; (2) mutagenicity assays in prokaryotes; (3) mutagenicity assays in eukaryotes; (4) tests for induction of DNA repair in eukaryotes; (5) tests for sister chromatid exchange; (6) cell transformation. Not all of these have equal sensitivity, specificity and reliability. The sequence of *in vitro* tests permits preliminary decision making. As a second series, limited, relatively rapid, *in vivo* assays involve the following: (1) skin-tumour induction in mice, with and without promotion; (2) lung-tumour induction in mice; (3) breast-cancer induction in rats; (4) identification of early lesions in rodent liver. The data so obtained are considered for decision making and risk analysis. As a last step, a traditional chronic bioassay may be needed only when human exposure to the product is potentially high and/or continuous, or when the above phases of testing have yielded unsatisfactory or, in the case of epigenetic agents, negative results. More research is essential for the delineation of the effects of epigenetic agents, some of which are most important in the aetiology of human cancer.

Introduction

Cancer is a general term for diverse diseases caused by many distinct but specific risk factors, namely different kinds of chemical carcinogens, co-carcinogens or promoters, and also various forms of radiation, and possibly viruses. It is important not only to define the risk factors but also to consider the actual or potential specific target organs in establishing approaches to qualitative and quantitative estimates of risk.

Much progress in understanding some of the actual causes of diverse cancers resulted when complex multifactorial causes were systematically evaluated in relation to a single, specific kind of cancer, such as cancer of the stomach or colon. It was found that the risk factors for diverse cancers were truly distinct (Fraumeni, 1975; Hiatt, Watson & Winsten, 1977; Higginson, 1979; Reddy, Cohen, McCoy, Hill, Weisburger & Wynder, 1980; Wynder & Hoffman, 1979). While chemicals have caused cancer in certain occupations (Saffiotti & Wagoner, 1976; Shubik, 1976), most types of cancer affecting the general public in various parts of the world are due to lifestyle factors that include cigarette smoking, and also specific macro- and micro-nutrients, and nutrition in general (Table 1). Occupational cancer fortunately affects only a small number of individuals, and current scientific and technical advances, especially through the methods discussed in this paper, should enable us to eliminate this kind of risk entirely.

Substantial progress has been made not only towards establishing the risk factors for specific kinds of cancer but in understanding the mechanisms of carcinogenesis (Brookes, 1980; Coon, Conney, Estabrook, Gelboin, Gillette & O'Brien, 1980; Emmelot & Kriek, 1979; Griffin & Shaw, 1979). Study into the nature of the carcinogenic process has permitted the rational classification of chemical carcinogens. The quantitative aspects for each class of chemical carcinogen, however, also need to be considered in more detail than has been done in the past (Weisburger & Fiala, 1981).

This paper presents the evidence for a new view of quantitative and qualitative distinctions in the mode of action associated with diverse chemical carcinogens. These concepts, in turn, bear directly on contemporary requirements for regulatory actions and risk analysis designed to minimize disease, and especially cancer, risks. It will be shown that chemical carcinogens can be divided into two broad categories—genotoxic carcinogens and epigenetic agents. These two categories are further divided into a total of eight subgroups (Table 2).

Risk evaluation for the genotoxic agents, by virtue of their specific mechanisms of action, must necessarily be different from such an evaluation for agents operating by epigenetic pathways. Genotoxic agents interact with DNA and genetic material (Fig. 1). Once cell duplication with the so-generated abnormal DNA has occurred, the reaction is translated into an irreversible alteration in DNA.

*Detailed treatment of this topic can be found in the references listed for Weisburger & Williams (1980) and Williams & Weisburger (1981).

Table 1. *Probable causes of main types of human cancer*

Cause of cancer	Type of cancer	Percentage of total cancer cases in USA	Estimated no. of new cases per year in USA
Occupational	Various	1–5	7850–39,000
Cryptogenic (viruses?)	Lymphomas, leukaemias, sarcomas, (cervix?)	10–15	78,500–118,000
Lifestyle:			
Tobacco related	Lung, pancreas, bladder, kidney	23	181,000
Diet related – nitrite/nitrate, low vitamin C, mycotoxin	Stomach, liver	4	35,000
– high fat, low fibre, broiled or fried foods	Large bowel, pancreas, prostate breast, uterus	43	339,000
Multifactorial			
Tobacco–alcohol	Oral cavity, oesophagus	4	34,000
Tobacco–asbestos, tobacco–mining, tobacco–uranium–radium	Lung, respiratory tract	5	40,000
Iatrogenic			
Radiation, drugs	Various	1	7850

In contrast, the action of agents that operate by epigenetic mechanisms, which are subject to much further research, usually requires their presence at high levels for a long time, and indeed is reversible up to a certain point. So far as is now known, their action is also tissue-specific. For example, bile acids are powerful promoters of colon cancer, but act as inhibitors when tested in the classic mouse-skin system (Reddy *et al.* 1980; Watanabe, Narisawa, Wong & Weisburger, 1978). Saccharin has been the subject

Table 2. *Classes of carcinogenic chemicals*

Type of carcinogenic chemical		Mode of action	Example
Genotoxic:	Direct-acting carcinogen	Electrophile, organic compound, genotoxic, interacts with DNA.	Ethyleneimine
	Procarcinogen	Requires conversion through metabolic activation by host or *in vitro* to direct-acting carcinogen.	Vinyl chloride, benzo[*a*]pyrene, 2-naphthylamine, *N*-nitrosodimethylamine
	Inorganic carcinogen	Not directly genotoxic; leads to changes in DNA by selective alteration in fidelity of DNA replication.	Nickel, chromium
Epigenetic:	Solid-stage carcinogen	Exact mechanism unknown; usually affects only mesenchymal cells and tissues; physical form vital.	Polymer or metal foils; asbestos
	Hormone	Usually not genotoxic; mainly alters endocrine system balance and differentiation; often acts as promoter.	Estradiol, diethylstilboestrol
	Immunosuppresor	Usually not genotoxic; mainly stimulates 'virally induced', transplanted, or metastatic neoplasms.	Azathioprine, antilymphocytic serum
	Co-carcinogen	Not genotoxic or carcinogenic, but enhances effect of direct-acting carcinogen or procarcinogen when given at the same time. May modify conversion of procarcinogen to direct-acting carcinogen.	Phorbol esters, pyrene, catechol, ethanol, *n*-dodecane, sulphur dioxide
	Promoter	Not genotoxic or carcinogenic, but enhances effect of direct-acting carcinogen or procarcinogen when given subsequently.	Phorbol esters, phenol, anthralin, bile acids, tryptophan metabolites, saccharin

of much study in the last 10 years. There is sound evidence that this agent belongs in the category of epigenetic agents and acts as a promoter for cancer of the urinary bladder (Cohen, Arai, Jacobs & Friedell, 1979; Hicks, 1980; Nakanishi, Hagiwara, Shibata, Imaida, Tatematsu & Ito, 1980; Weisburger, 1980). Therefore, attempts to use standard techniques of risk analysis for saccharin have yielded controversial results because such approaches do not apply to this class of agent. In fact, new procedures to define the mode of action of epigenetic carcinogens, and hence risk evaluation, need to be developed.

Thus, current concepts of the mechanisms of carcinogenesis and the above classification of chemical carcinogens have been the basis of a rational sequential system of detecting carcinogens that might present a health risk. We hope that the detection of potential, but as yet unknown, future health risks, as well as the acquisition of knowledge of the health risks associated with and responsible for diverse kinds of existing important human cancers, will be simplified, accelerated and made more economical and reliable by use of such a systematic 'decision-point approach'.

The decision-point approach

The decision-point approach involves five sequential steps in the evaluation of the potential risks associated with the carcinogenicity of chemicals (Table 3). This approach was formulated to incorporate several newer developments in chemical carcinogenesis. Of major relevance was the view that chemicals

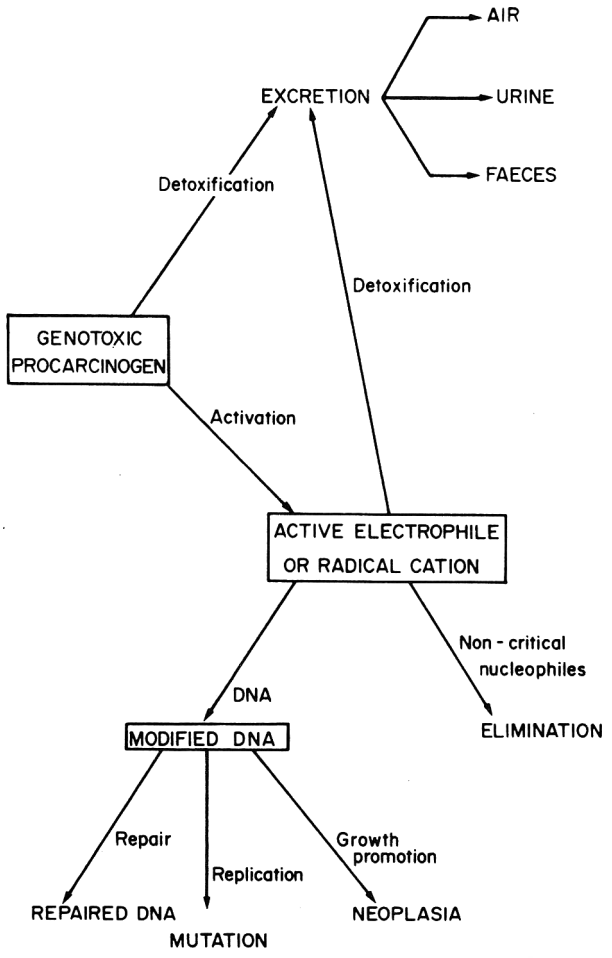

Fig. 1. Schematic presentation of the diverse biochemical reactions involved in the metabolic activation and detoxification of genotoxic carcinogens. For many carcinogens there are reactions leading to detoxified metabolites facilitating excretion. Usually only a small fraction of a dose of a carcinogen is converted, in one or more steps, to more toxic and carcinogenic metabolites, which are reactive electrophiles or radical cations. These metabolites can undergo enzymic detoxification reactions yielding excretable products, or they can react with nucleophiles that are non-critical to the carcinogenic or mutagenic process in competition with the critical nucleophiles, especially DNA. *In vitro* systems have different ratios of activation:detoxification enzymes and distinct levels of non-critical nucleophiles, and thus account for differences found between *in vivo* and *in vitro* assays. The key reaction is with DNA, providing a parallel between mutagenesis and carcinogenesis. This lesion can be repaired, an effect that plays a role in quantitative carcinogenesis and in the specific organ primarily affected by a given carcinogen. Essential elements in the overall process towards mutation or neoplasia are DNA and cell duplication, growth, and promotion. [Based on a figure from Weisburger & Williams (1981).]

Table 3. *Decision-point approach to carcinogen testing*

Stage	Action
A	Consideration of the structure of the chemical
B	Battery of *in vitro* short-term tests
	(1) Bacterial mutagenesis
	(2) Mammalian cell mutagenesis
	(3) Mammalian cell DNA repair
	(4) Mammalian cell chromosome effects
	(5) Mammalian cell transformation
C	Limited *in vivo* bioassays
	(1) Skin-tumour induction in mice
	(2) Pulmonary-tumour induction in mice
	(3) Breast-cancer induction in rats
	(4) Induction of rodent liver altered foci
	(5) Assays for promoters
D	Chronic bioassay
E	Final evaluation

could produce an increase in the tumour incidence in exposed animals, i.e. be carcinogenic, by several distinct mechanisms, each having different theoretical and practical implications. One of these mechanisms, proposed by Drs Elizabeth and James Miller, was through the generation of an electrophilic reactant that could react covalently with cellular macromolecules. Research in a number of laboratories, notably those of the Millers, Brookes and Lawley, Magee and Swann, and Ames (see Brookes, 1980; Grover, 1979; Miller & Miller, 1979; Weisburger & Williams, 1980) has strongly indicated that DNA is in fact the critical cellular target. However, in addition to chemicals that react with DNA, others lacking this property are nevertheless carcinogenic or oncogenic under certain conditions. Among chemicals of the latter type are plastics, hormones, immunosuppressants, cytotoxic agents, co-carcinogens and promoters. Thus, we suggested that chemical carcinogens could be divided into two main categories, on the basis of their capacity to damage DNA (Table 2).

Carcinogens that reacted covalently with DNA were classified as genotoxic, a term first used by Druckrey (see Ehrenberg, Brookes, Druckrey, Lagerlof, Litwin & Williams, 1973), while those lacking this property and probably acting by other mechanisms, were categorized as epigenetic (Weisburger & Williams, 1980). The genotoxic category contains the classic organic carcinogens that damage DNA either through direct chemical reactivity or following metabolism by enzyme systems. In addition, in the light of some evidence for DNA damage or alteration during biosynthesis by inorganic carcinogens, these were tentatively placed in this category.

In contrast, the second category of epigenetic carcinogens is composed of those agents that have not been found to damage DNA, but rather appear to act through other indirect mechanisms. This category contains several classes of agents that operate by distinct non-genotoxic mechanisms, mainly affecting a specific target organ.

Stage A. Structure of the chemical

The first step in evaluating possible carcinogenicity is a consideration of the chemical structure. For specific classes of chemicals such as aliphatic hydrocarbons, polycyclic aromatic hydrocarbons, aromatic amines, and aliphatic or cyclic nitrosamines, sufficient experience has accumulated on structure *versus* carcinogenicity to develop preliminary information on potential carcinogenicity on the basis of the structure of the chemical itself and its potential metabolites. There are species-linked differences in metabolism that bear on carcinogenic risks.

Information on structure and metabolism provides a guide to the selection of specific limited bioassays at stage C (below), and as more information becomes available, may also contribute to the selection of relevant short-term tests at stage B.

Stage B. In vitro *short-term tests*

There is general agreement that a battery of such assays is necessary rather than a single test (see Williams, Kroes, Waaijers & van de Poll, 1980). Therefore, the key element in developing a battery of tests is to formulate suitable criteria for selecting the most effective and economical combination of tests. Furthermore, since such testing is becoming quite complex and expensive, it is relevant to attempt to specify the number of tests that are essential without loss of information. The basis for the selection of *in vitro* tests has been reviewed in detail (Williams & Weisburger, 1981).

At this time, a suitable screen necessarily includes microbial mutagenicity tests as developed by Ames, Rosenkranz, Sugimura, Malling, DeSerres, and others. Especially useful are Ames' test involving *Salmonella typhimurium*, and variations thereof (Ames & Haroun, 1980). Mammalian cell mutagenicity tests are included because of the significance of mutagenic effects in the eukaryotic genome, which differs in several important respects from the prokaryotic genome. DNA repair is a specific response to DNA damage, and thus, a test based on this phenomenon provides an endpoint that is highly specific and of great biological significance. A chromosomal test is useful in the battery to delineate chemical effects at a higher level of genetic organization. Cell transformation needs consideration because this test possibly shares mechanisms with those prevailing for *in vivo* carcinogenesis.

In summary, the battery includes tests for (1) bacterial mutagenesis, (2) mammalian mutagenicity, (3) DNA repair, (4) chromosomal effects and (5) cell transformation. All of these tests need elaboration to denote their advantages and especially their limitations.

The results of these tests and structure–activity relationships should be used for preliminary decision making. A positive response in one or more tests indicates suspicion of genotoxicity. This then can be explored further with specifically designed limited *in vivo* bioassays (stage C).

If the *in vitro* test systems yield no evidence of genotoxicity, the priority for further testing depends on two criteria: (1) the structure and known physiological properties (for example, hormonal) of the material; (2) the potential human exposure.

Stage C. Limited in vivo *bioassays*

This stage involves tests that underline available evidence of the hazard of chemicals positive for geno-

toxicity, and yet avoids a long-term chronic bioassay without much loss of convincing data. These tests include: (1) skin-tumour induction in mice, with and without promotion, and with and without initiation, thus giving information on possible initiating or promoting activity of the chemical; (2) pulmonary-tumour induction in mice; (3) breast-cancer induction in female Sprague–Dawley rats; (4) altered foci induction in rodent liver, and (5) variations involving initiators and promoters.

The preceding tests have an advantage in that they truly represent *in vivo* carcinogenesis bioassay. Also, they yield results in less than a year, and in some instances in less than 6 months. Some yield multiple tumours, and thus provide useful semi-quantitative comparative information. Not all classes of carcinogens give positive results in any one of these tests. However, a definite positive result and evidence of genotoxicity from the battery of *in vitro* tests supports the view that a chemical substance or mixture would be carcinogenic. A negative result in any one of the limited bioassays would not necessarily rule out carcinogenic potential. The classes of compounds active in such limited bioassays have been delineated (Williams & Weisburger, 1981).

Stage D. Chronic bioassays

Chronic bioassay is used in the decision-point approach as a last resort for confirming questionable results in the more limited testing, for compounds that are negative in the preceding stages of testing but to which extensive human exposure is likely, or for the acquisition of data on possible carcinogenicity through epigenetic mechanisms. In the latter situation, multi-species and dose-response data are most important, if the results are to be applied meaningfully to risk assessment. Also, if a specific target organ is suspected of being involved, a limited pretreatment with a known genotoxic carcinogen for that organ would facilitate detection and delineation of the effect of the epigenetic agent.

Stage E. Final evaluation

If the decision point approach has led to a chronic bioassay, then fairly definitive data on carcinogenicity would be obtained. However, the results of the *in vitro* short-term tests together with consideration of the structure of the compound must be incorporated into an evaluation of possible mechanisms of action and risk extrapolation to humans. Convincing positive results in the *in vitro* tests coupled with documented *in vivo* carcinogenicity permits classification of the chemical as a genotoxic carcinogen.

If, on the other hand, no convincing evidence for genotoxicity is obtained, but the chemical substance is carcinogenic in certain animal bioassays, then the possibility exists that the chemical is an epigenetic carcinogen. The strength of this conclusion depends upon the structure of the compound and the relevance of the *in vitro* tests.

Quantitative aspects

It is important to realize that health-risk analysis must consider quantitative potential as well as qualitative positive or negative results. It is evident that distinctly different protective measures are needed for the liver carcinogen aflatoxin B_1 (active at 1 ppb*), than for the liver carcinogen safrole (active at 2000 ppm) or acetamide (active at 12,500 ppm). With the powerfully carcinogenic *N*-2-fluorenylacetamide, a lowering of the dose by only one log unit, a factor of 10, converts a very powerful carcinogenic stimulus (200 ppm) to a virtually inactive dose rate (20 ppm). On a larger scale, in the case of cigarette smoke, an individual smoking 40 standard cigarettes per day has a fairly high risk of disease, whereas four cigarettes per day would be a minimal risk. This again is only a factor of ten. Thus, quantitative aspects are most important if the goal of risk elimination, and thus disease prevention, is to be approached in a realistic manner.

Conclusion

The decision-point approach provides a framework, based on current concepts of the mechanisms of carcinogenesis, for the systematic evaluation of the potential mutagenic and carcinogenic hazards of chemicals. This approach can be integrated with other elements in toxicity testing. It is designed to yield a stepwise progression of data acquisition. A carefully conducted evaluation, based on this systematic programme should provide a qualitative and a semi-quantitative sequential data base, and need not necessarily terminate in an expensive and extensive long-term bioassay. This approach provides an effective tool for the protection of the public against environmental carcinogenic and mutagenic factors through health-risk analysis, based on current concepts of risk extrapolation to humans. Convincing positive results in the *in vitro* tests coupled with documented *in vivo* carcinogenicity permits classification of the chemical substance as a genotoxic carcinogen. At that point, an expert group needs to consider whether the value of such a material to human beings at the potential maximal exposure levels is sufficient to tolerate possible human exposure. This risk analysis needs to consider the relative strength of the carcinogen. In the USA, such a value judgment has been made, for example, with respect to the potential risk attached to the powerful carcinogen aflatoxin B_1 as was discussed previously in this paper.

Much more research is necessary on the mode of action of epigenetic agents (see Williams (1981) for a discussion of promoters). Nonetheless, it would appear that risk analysis for such materials will show that the action of such agents will present a definite threshold which can be determined experimentally. Thus, the hazards of human exposure may be mainly of a quantitative nature. Furthermore, in evaluating the effects of intermittent use or exposure to such agents it should be borne in mind that their action is reversible up to a point.

In summary, the proposed testing approach, which embodies new concepts in chemical carcinogenesis, provides for systematic yet economical testing, the results of which can be applied to flexible, rational health-risk analysis.

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*b = 10^9.

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GENETIC AND NONGENETIC EVENTS IN NEOPLASIA

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Summary—It has become increasingly evident that all chemical carcinogens do not act *via* the same mechanism of tumorigenicity. Based upon the extent of a chemical's interaction with DNA, a general classification scheme of various mutational and nonmutational theories of chemical carcinogenesis is presented. Compounds that directly interact with DNA are classified as genotoxic whereas those that do not interact directly with DNA are classified as epigenetic carcinogens. Under each general heading, several mutational and nonmutational mechanisms of carcinogenesis are believed to be possible. Data are also presented to support the existence of one such mechanism, an epigenetic-mutational theory of chemical carcinogenesis based upon recurrent cytotoxicity. In this case, increased regenerative DNA synthesis in response to tissue injury is believed to result in an enhancement of the normal spontaneous mutation rate, conceivably leading to a cellular transformation. The carcinogenic risk posed by such epigenetic carcinogens appears to differ greatly from that posed by genotoxic carcinogens. Thus, consideration of data concerning the possible mechanism of carcinogenicity of a chemical, along with pharmacokinetic data, will allow a better understanding of bioassay results and a more accurate assessment of carcinogenic risk.

Introduction

Frequently in the routine rodent carcinogenesis bioassay of a chemical high levels of a test compound are administered for prolonged periods of time and, at the termination of the study, the numbers of tumours formed are counted. If a statistically significant excess of a particular tumour is observed the compound is classified as an animal carcinogen and an extrapolation of risk is made to humans. Yet in most cases, without knowledge of the fate of the chemical in several species (i.e. pharmacokinetics) as well as of the resultant macromolecular events to help understanding of the bioassay results, there is little chance of a meaningful assessment of human carcinogenic risk. It is the integration of these three data bases (bioassay, pharmacokinetics and macromolecular events) to more realistically assess the potential carcinogenic risk of a given chemical that has been a continuing endeavour in our laboratory over the past several years. Recently, we have been involved in trying to understand the different mechanisms of tumour formation and their implications for risk assessment, and, more specifically, the role of cytotoxicity in the carcinogenic process. This latter area of research will be the focus of this paper.

Classification of proposed mechanisms of chemical carcinogenesis

Since the first experimental documentation of chemically induced carcinogenesis in animals by Yamagiwa & Ichikawa (1918), numerous theories have been proposed to explain the mechanisms of chemical carcinogenesis. Apart from the various differentiation, protovirus and viral infection theories of carcinogenesis (Temin, 1974), most theories regarding the mechanism(s) of chemical carcinogenesis may be generally classified as 'genetic' or 'epigenetic' (nongenetic) in nature. As discussed below, the distinction between these two general classes of mechanisms is of great significance in carcinogenic risk assessment. As used here, chemicals displaying a genetic mechanism of carcinogenesis are defined as those involved in direct interaction with cellular DNA. In most cases this interaction is considered to result in a mutational event; however there may also be cases of nonmutational genetic mechanisms of carcinogenesis (e.g. Holliday, 1979). Chemicals displaying an epigenetic mechanism of tumorigenesis are defined as those that do not directly interact with cellular DNA, and yet their action may indirectly result in a mutational event. Thus, the terms genetic and epigenetic in chemical carcinogenesis, as used here, refer to the likelihood of direct chemical–DNA interaction, and in either case may or may not involve a mutational event.

The most common interpretation of a genetic mechanism of tumorigenesis is embodied in the somatic mutation theory of carcinogenesis first proposed by Boveri (1929). Basically, this theory dictates that the direct interaction of a chemical with DNA (e.g. by alkylation, intercalation) can result in a somatic cell mutation (*via* point or deletion mutation, gene duplication or gross chromosomal disruptions) which may or may not ultimately lead to a transformed or neoplastic cell. As reviewed by Hanawalt, Friedberg & Fox (1978) mitigating factors in this progression are the DNA-repair enzymes which remove the chemically induced 'lesion' before (excision repair) or after (post-replication repair) DNA replication (i.e. before the lesion is fixed as a mutation). It also appears that errors in this repair, possibly by an inducible error-prone DNA-repair enzyme system analogous to SOS repair observed in bacteria, may be responsible for the mutation rather than the initial chemically-induced mispairing of bases (Kondo, 1976 & 1977; San & Stich, 1975; Sarasin & Benoit, 1980; Witkin, 1976).

Comings' (1973) 'general theory of carcinogenesis' provides a possible scenario of events leading to a state of anaplasia after a mutation occurs. In this case the significant events would be a double mutation of one or more regulatory genes (normally present in all cells), in turn derepressing corresponding structural genes capable of coding for cellular transforming factors. In the case of chemical carcinogenesis following an apparent two-step progression towards neoplasia ('two-step theory of carcinogenesis'). this derepression of genes following a mutational event (initiation) may be promoted by epigenetic stimuli, chemical or physical (Boutwell, 1974; Trosko & Chang, 1978).

Substantial evidence has been accumulated to link mutagenic events with the neoplastic transformation of cells. As reviewed by Trosko & Chang (1978), findings regarding the clonal nature of tumours, the mutagenicity of many carcinogens, the correlation of high cellular mutation frequency with increased cancer rates among humans suffering from deficiencies in DNA-repair enzymes, the correlation of *in vitro* DNA damage with cell transformation frequencies and neoplasia, the involvement of mutation in the initiation phase of some hydrocarbon-induced carcinogenesis, the effects of age on mutagenesis and the incidence of various hereditary tumours, are all supportive of a mutagenic origin of cancer. However, several findings cannot be reconciled with a mutagenic basis of carcinogenesis. These have included observations regarding the fact that all carcinogens are not mutagens (Jensen, 1974: Lippman, 1975; Segaloff, 1975), the discrepancy between gene mutations and cell transformation rates in short- *v.* long-lived animals (Huberman, Mager & Sachs, 1976; Peto, 1977), the induction of tumours by plastics- or metal-film implantation and hormone imbalance (Berenblum, 1978), the ability of teratoma cells to produce apparently normal cells upon transplantation into normal mosaic mouse blastocytes (Mintz & Illmensee, 1975; Papaioannou, McBurney, Gardner & Evans, 1975) and the ability of Lucké frog adenocarcinoma cells to reproduce normal cells upon nuclear transplantation into anucleate eggs indicating a totipotency of the tumour-cell genes (McKinnell, Deggins & Labat, 1969). Thus, there is also substantial evidence for the existence of one or more epigenetic nonmutational mechanism of carcinogenesis.

Several theories have attempted to reconcile these different mechanisms of carcinogenesis either by proposing an alternative to mutagenesis or by accommodating both genetic mutational and epigenetic nonmutational mechanisms. A recent example of the former situation is the nonmutagenic theory proposed by Holliday (1979). This theory proposes that gene regulation, as directed by specific DNA base methylation and recognition by several DNA methylases, may be altered during repair of DNA–chemical alkylation sites. The resultant loss of specific methylated recognition sites for DNA methylases results in loss of gene regulatory control and ultimately in a transformed cell. Since the direct interaction of the chemical with the DNA is required to deregulate genes in this nonmutagenic theory, it is still consistent with a genetic mechanism of tumorigenicity as defined above. An example of the latter 'coexistence' type of theory is the 'integrative theory' of carcinogenesis of Trosko & Chang (1978). These authors propose that neoplasms may arise from a mutagenic event alone (regulatory locus) if the genes affected are in a transcribable state, from a mutagenic event if there is a coupled nonmutational alteration of the mutated genes (promotion), or from the abnormal derepression of genes at critical developmental stages that prevent normal gene regulation.

Based upon the apparent realization that some chemicals may cause tumours *via* several different mechanisms of action (i.e. there is no single unifying mechanism), it has been possible to propose a classification scheme for chemical carcinogens even though mechanistic details remain to be elucidated. Weisburger & Williams (1980) have thus proposed that carcinogenic chemicals be categorized under the general division of genotoxic chemicals (direct-acting or primary carcinogens, procarcinogens or secondary carcinogens, inorganic carcinogens) and epigenetic carcinogens (solid-state carcinogens, hormones, immunosupressive agents, co-carcinogens, promoters). A diagrammatic representation of the various theories of carcinogenesis is presented in Fig. 1.

Concept of the cytotoxic mechanism of tumour formation

An epigenetic mechanism of tumorigenesis that is not frequently considered in terms of tumour production is that of recurrent cytotoxicity. The simplest example of this mechanism is the production of sarcomas following subcutaneous injection of inert solutions such as saline or glucose. It is envisaged that repeated subcutaneous injection of these nonreactive materials results in inflammation, necrosis and cellular division which may increase the error rate in normal DNA replication or DNA repair, resulting in critical-site mutation and ultimately in a transformed or neoplastic cell. In general terms, this theory represents a modification of Virchow's 'irritation theory' in which hyperplasia was believed to be the driving force behind carcinogenesis (Berenblum, 1944). It is likely that some chemicals may illustrate both genetic and epigenetic effects. In such an instance it will be important to determine which mechanism is predominantly responsible for the formation of tumours.

With the concept of testing maximum doses in animals in order to enhance the probability of detecting a carcinogenic response, one of the primary issues is the relevance of such testing in terms of the real risk for humans of a chemical shown to be carcinogenic under such test conditions. Frequently in such studies tumours are observed at dose levels that also cause recurrent cytotoxic responses, and a logical question would be whether an analogy to sarcoma production following subcutaneous injection may be made. In understanding this relationship, it is enlightening to explore the molecular basis for increasing mutation as a consequence of increased DNA synthesis and cell division.

DNA does not exist *in vivo* as a pristine molecule even in the absence of a measurable genotoxic challenge. So-called spontaneous mutations appear to occur at a rate dependent upon exposure to unavoidable exogenous (e.g. cosmic radiation) and endogen-

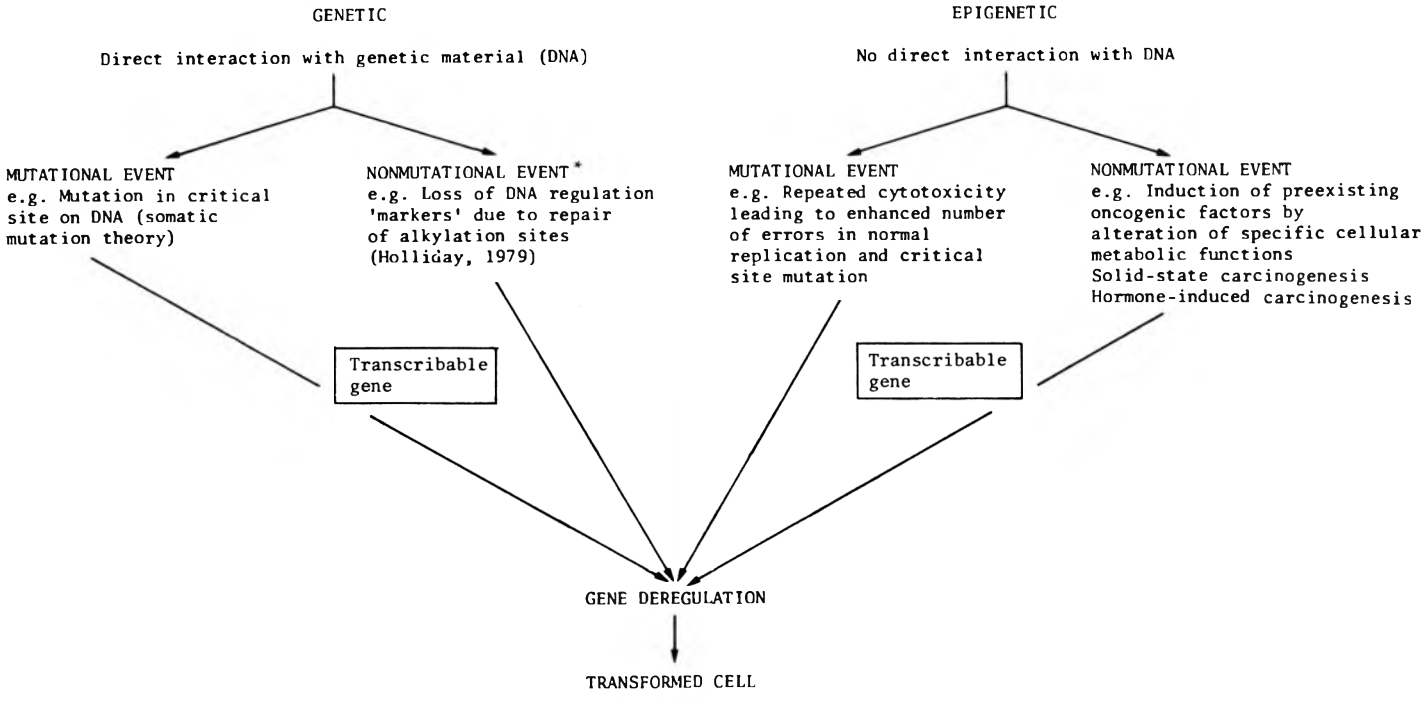

Fig. 1. General classification of some of the proposed mechanisms of carcinogenesis, excluding the differentiation, oncogenic, viral infection and protovirus theories. The role of DNA repair in mutagenesis is not included.
*Believed to be a readily reversible alteration (Holliday, 1979).

ous factors, DNA-repair competency (e.g. relative to age) and the basic thermo-stability of the DNA molecule itself. With regard to the latter point it has been estimated that thermal decomposition may account for between 2000 and 10,000 depurinations, several hundred depyrimidinations and several base deaminations of DNA molecules in a mammalian cell per generation (Lindahl & Karlstrom, 1973; Lindahl & Nyberg, 1972 & 1974). These thermal lesions can result in base transitions (e.g. deamination of 5-methylcytosine to form thymine) which conceivably may be 'fixed' (irreparable) by DNA replication or repair processes resulting in a functional mutation. Indeed, errors in the process of DNA replication itself may occur as a result of base mispairing, DNA polymerase base selection errors and mismatch repair errors (see review by Hartman, 1980). Based upon estimates of the mutation rates per human gene per sexual generation, it has been suggested that 10% of all human gametes contain a new mutation of their own plus several inherited mutated genes as well (Drake, 1978). Thus the mere fact that DNA synthesis and cellular division is enhanced following cytotoxic responses will result in a decreased time for repair of 'naturally' occurring DNA lesions before replication, an increased number of replication errors, and an enhanced somatic mutation rate.

Additionally, since errors in DNA may occur throughout the cell cycle or during DNA synthesis, the DNA-repair process can be thought of as a continuous process. However, as noted, some forms of DNA repair are error-free while others may be error-prone. Therefore the fidelity of DNA repair can affect the somatic mutation rate during cell division. A recent report by Shank & Barrows (1980) on studies of the noncarbonatious chemical hydrazine has shown that cytotoxic doses of hydrazine cause abnormal methylation of nucleic acid bases in the liver. Furthermore, evidence was presented which suggests that at hepatotoxic doses other chemicals (e.g. carbon tetrachloride) produce a similar alteration in the DNA as a result of cytotoxicity. Whether this effect is due to an alteration in the DNA polymerase or in the fidelity of DNA repair following DNA replication is not known, but it is an example of how a cytotoxic agent at high dose levels can cause abnormal nucleic acid bases to occur in cellular DNA.

DNA synthesis and cell division can also enhance the susceptibility to mutation by exogenous and endogenous agents. For example, Berman, Tong & Williams (1978) have reported an increased mutation frequency in actively dividing adult rat-liver epithelial cells exposed to the mutagenic chemical methyl methanesulphonate compared with that in quiescent (nondividing) cells. In this study the number of azaguanine resistant colonies per 10^6 colony-forming units was used as a measure of mutation frequency. The mutation frequency in the actively dividing cultures was increased five-fold when compared with the quiescent culture, indicating that dividing cells were more susceptible to mutation.

Finally, DNA synthesis and cell division can also enhance mutation frequency by altering the amount of DNA repair prior to DNA replication. An example of this is given by the work of Maher, Dorney, Mendrala, Konze-Thomas & McCormick (1979) using normal human fibroblasts and fibroblasts lacking normal DNA excision repair. Cell survival and mutation rate in cells exposed to ultraviolet (UV) radiation was observed to be related to DNA-repair competency. Survival of normal cells was higher and mutation rate lower per UV dose than for deficient cells. Normal cells were also able to survive a usually lethal and mutagenic UV dose by being held in confluence (nondividing) for a period of time before assessment of survival and mutation rate, presumably because this allowed a greater time for DNA repair to occur before replication.

In addition to a molecular basis for enhancing mutagenic events as a result of increased DNA synthesis, there is an experimental basis for the role of DNA synthesis in carcinogenesis. Tumours often develop in chronically inflamed or scarred tissue; colon cancer is frequently observed in patients with chronic colitis; skin cancer occurs in burn scars; liver tumours are associated with chronic cirrhosis of the liver (Berenblum, 1944; Chan, 1975; Laroye, 1974). Repeated tissue damage with a physical agent (dry ice) and resultant regeneration has induced tumours in mice (Berenblum, 1929). Physical trauma such as partial hepatectomy has also been shown to enhance the tumorigenic effect of thioacetamide, *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethylamine (Craddock, 1978; Date, Gotoskar & Bhide, 1976). Dimethylbenzanthracene-induced skin tumours promoted by the classic phorbol esters have been observed to be inhibited by anti-inflammatory steroids which inhibit inflammation, DNA synthesis and cell proliferation (Slaga, Fisher, Viaje, Berry, Bracken, LeClerc & Miller, 1978; Weeks, Slaga, Hennings, Gleason & Bracken, 1979). Finally, as noted above, tumours can be induced at the site of subcutaneous injection of nonreactive chemicals, such as glucose, saline or distilled water (Grasso & Golberg, 1966).

Having explored the molecular as well as experimental basis for the effects of cytotoxic responses on the carcinogenic process, the obvious question arises about the impact of cell division and increased DNA synthesis on genotoxic chemical carcinogens. For genotoxic carcinogens it is likely that cytotoxicity will cause an increased incidence and/or a decreased latency in the production of tumours. A good example of this is the induction of liver tumours by NDMA (Terracini, Magee & Barnes, 1967). At low doses of NDMA that preclude cytotoxic effects in the liver, tumours can still be induced. At higher levels where both cytotoxic as well as genotoxic effects act in concert, liver tumours are observed at an increased frequency as well as at a decreased latency. For chemical carcinogens displaying a cytotoxic mechanism with little to no genotoxic activity it is likely that a prolonged, recurrent cytotoxic response throughout a large portion of the animal's life will be required for the induction of tumours.

The implications of these carcinogenic mechanisms are as follows. (1) For genotoxic carcinogens, the defence mechanisms such as DNA repair, detoxification and excretion of the reactive species will be critical for modifying the carcinogenic response. Risk estimates need to consider the dose-related kinetics of the lesions produced and the extent and persistence of those genetic lesions. (2) For epigenetic carcinogens

causing tumours *via* a cytotoxic mechanism of tumorigenicity, tumours will be induced at doses that produce recurrent cytotoxicity and no carcinogenic risk would be predicted at doses that preclude any tissue injury.

It is expected that chemical carcinogens will represent a spectrum of activity ranging from genotoxic to epigenetic. Those chemicals having strictly genetic activity are often those that either are, or can be activated to, strongly electrophilic species. Chemicals with intermediate activity may have both a genetic as well as an epigenetic component to their tumorigenic response and some chemicals that show little to no genetic activity may produce tumours strictly through the mechanism of recurrent cytotoxicity or other epigenetic mechanisms. This is not to say that chemical reactivity is always directly relatable to carcinogenic potency but only that such compounds show a propensity to react with macromolecules. Obviously, the qualitative character or reaction site specificity of these reactions will have a profound impact upon the outcome of the DNA–chemical interaction. Indeed, a majority of interactions would not be expected to result in any biologically significant event because they involve noncritical-site alkylation or repair. It is also important not to rule out endogenous factors such as genetic predisposition working in concert with epigenetic mechanisms since all species and strains are susceptible to the development of particular tumours that are not related to exogenous chemical exposure. This concept is of paramount importance in assessing the relevance of extrapolating test results in sensitive animal species to humans.

Experimental approaches: some examples

The experimental approach taken in our laboratory has been to develop some objective criteria to differentiate between the genetic effect and the epigenetic, cytotoxic mechanism of tumour production. The two parameters that were selected as representative of a genetic effect were (1) DNA damage, measured by alkylation of DNA *in vivo* and (2) DNA repair, induced presumably by damage to the DNA molecule. While there are inherent limitations to using total DNA alkylation as an index of genetic interaction, due to the overall lack of information on specific critical sites of reaction on all of the nucleic acid bases, it was one of the only means of assessing the potential for *in vivo* genetic interaction. NDMA was selected as a genetic-acting carcinogen and the extent of DNA alkylation and of DNA repair following treatment with the test chemicals were compared with those after NDMA treatment. NDMA was also selected because it represented an electrophilic methylating agent and the chemicals we were testing were low-molecular-weight molecules. If high-molecular-weight heteroaromatic molecules are being tested, benzo[*a*]pyrene or the naphthylamines may be appropriate. For evidence of epigenetic, cytotoxic effects, DNA synthesis in the target tissue was measured and classic histopathology was also carried out. The chemicals that were tested were perchloroethylene, chloroform and 1,4-dioxane,

The results of the carcinogen bioassay on perchloroethylene conducted by the National Cancer Institute and of studies conducted in our laboratory are summarized as follows. Mice exposed by gavage to approximately 500 or 1000 mg/kg/day throughout their lifetime showed an increase in liver tumours (hepatocellular carcinomas), but rats exposed to equally high doses did not show any tumorigenic response (National Cancer Institute, 1977). In inhalation studies, exposure to 300 or 600 ppm perchloroethylene daily for 1 yr (subsequent observation for 18 months) did not prove to be tumorigenic in rats (Rampy, Quast, Leong & Gehring, 1978). It is important to emphasize that the $B6C3F_1$ mice in which liver tumours were enhanced by perchloroethylene treatment have an average control incidence of hepatocellular carcinoma of 10–15% (some nearly 50%). Therefore, this species and strain has a marked genetic predisposition for liver neoplasms.

Studies designed to characterize differences in the pharmacokinetics of perchloroethylene in rats and mice showed the following (Table 1; Schumann, Quast & Watanabe, 1980; Watanabe, Reitz, Schumann, McKenna, Quast & Gehring, 1980). When exposed to a 10 ppm perchloroethylene atmosphere for 6 hr mice metabolized and activated nine times more perchloroethylene than did rats. This resulted in a seven-fold greater macromolecular binding in the liver of mice than rats. Importantly, binding to hepatic DNA in mice at a tumorigenic dose level of 500 mg/kg was not observed. The detection limit for the perchloroethylene–DNA binding studies in mice was 10 alkylations per 10^6 nucleotides.

When parameters of cytotoxicity were assessed (Table 1) the liver weights were increased in treated mice but not in rats. DNA synthesis caused by tissue injury was increased by 82% in the mice but was not significantly increased in rats, and finally histopathological alterations in the liver were evident in mice but not in rats.

Another possible indication of direct genetic interaction is that of gross chromosomal damage and of *in vitro* mutagenesis, if a possible nonmutational gene deregulation mechanism of carcinogenesis (e.g. Holliday's theory) is ruled out. When mutagenicity data for perchloroethylene were examined (Table 2), it was noted that negative results in Salmonella have been reported from three laboratories and there has been one report of a positive finding in the Ames-type strain of *Salmonella typhimurium*. Further, in a cytogenetics study no chromosomal abnormalities attributable to perchloroethylene exposure were observed. The overall conclusion is that perchloroethylene has little to no genetic activity.

In summary of the perchloroethylene data, DNA alkylation was not detectable at a detection limit of ten alkylations per 10^6 nucleotides; DNA-repair assays were not conducted because they are generally less sensitive than the alkylation studies. Mutagenicity data is predominantly negative in bacterial systems as well as negative in an animal cytogenetics study. Cytotoxic responses indicated by increased DNA synthesis, two-fold over that of control, and histopathological examination were evident in the mouse at tumorigenic dose levels. In contrast, a classic genotoxic carcinogenic agent (e.g. NDMA, ethylnitrosourea) would alkylate DNA at a level of hundreds to thousands per 10^6 nucleotides; DNA repair would

Table 1. *Perchloroethylene metabolism, genotoxicity and cytotoxicity data (Schumann, Quast & Watanabe, 1980; Watanabe, Reitz, Schumann, McKenna & Gehring, 1980)*

Perchloroethylene treatment	Parameter	Response in Rats	Response in Mice
Inhalation exposure to 10 ppm for 6 hr	Total metabolized (μmol-equiv/kg body weight)	10·5	89·5
	Total macromolecular binding (μmol-equiv/g hepatic protein)	0·02	0·147
Single oral dose of 500 mg/kg body weight	Hepatic DNA binding	—	ND
Twelve oral doses of 500 mg/kg body weight/day	Liver weight:body weight (% increase)	5	25
	Hepatic DNA synthesis (% increase)*		
	Hepatic histopathological changes†	−	+

ND = Not detected

*Present as a percentage increase in DNA synthesis in treated compared with control animals as measured by [6-^{3}H]thymidine uptake.

†A treatment-related response was observed (+), or no treatment-related response was observed (−).

illustrate a dose-response relationship; *in vitro* mutagenicity would be clearly evident. In contrast to these genotoxic effects, there would be little histopathological alteration or increased DNA synthesis in the target organs at low, though tumorigenic, dosages, thus providing evidence of a lack of a significant role of an epigenetic, cytotoxic mechanism. Our overall conclusion is that the liver tumours observed in mice following lifetime administration of high doses of perchloroethylene were a result of the recurrent hepatotoxic effect of perchloroethylene which caused an enhancement of the normal background liver tumour incidence in a strain of mice with genetic predisposition towards the development of such tumours. Most importantly, hepatotoxic effects in animals and humans would be observed only at dose levels that are well above levels encountered in the occupational environment. Thus the relevance of the data that has been obtained for mice which suggests a carcinogenic risk to humans exposed at occupational or environmental levels of perchloroethylene is highly questionable.

The next example is that of chloroform (Reitz, Quast, Stott, Watanabe & Gehring, 1980). The carcinogenicity data for male mice given chloroform are summarized in Table 3. This represents a compilation of more than one study, but these data clearly show that following administration of chloroform at high doses male mice are susceptible to the development of liver and kidney tumours. It is noteworthy that at 17 mg/kg/day no significant incidence of tumours at any site was observed. At high doses chloroform also induces tumours in the rat, but for the sake of space we shall limit discussion here to mouse data.

At an oral dose level of 240 mg chloroform/kg body weight, mouse liver and kidney DNA was alkylated at a rate of three and one alkylations per 10^6 nucleotides respectively (Table 4). These values represent the upper bounds of chloroform alkylation since no actual DNA adducts have been identified. In contrast,

Table 2. In vitro *mutagenicity data and cytogenetics data on perchloroethylene*

Bioassay system	Results*	Reference
Escherichia coli	−	Greim, Bonse, Radwan, Reichert & Henschler (1975)
Salmonella strains	−	National Institute for Occupational Safety and Health (1977).
	+	Černá & Kypěnová (1977)
	−	Bartsch, Malaveille, Barbin & Planche (1979)
	−	National Toxicology Program (1980)
Cytogenetics	−	Černá & Kypěnová (1977)

*Positive (+) and negative (−) responses are indicated.

Table 3. *Data from tumorigenicity assays of chloroform given orally to male mice*

Chloroform dose	Strain	Percentage of excess tumours (site)	Reference
277 mg/kg/day, 5 days/wk for 78 wk	$B6C3F_1$	92 (liver)	National Cancer Institute (1976)
60 mg/kg/day, 6 days/wk for 80 wk	C57BL	ND	Roe, Palmer, Worden & Van Abbé (1979)
60 mg/kg/day, 6 days/wk for 80 wk	ICI	23 (kidney)	Roe *et al.* (1979)
17 mg/kg/day, 6 days/wk for 80 wk	ICI	ND	Roe *et al.* (1979)

ND = Not detected

a dose of 10 mg NDMA/kg results in approximately 540 alkylations per 10^6 nucleotides in the liver. Similarly, a tumorigenic dose of 240 mg chloroform/kg was not observed to induce hydroxyurea-resistant DNA repair (Table 4) while NDMA showed a dose-related increase in DNA repair following doses of 3, 10 and 20 mg/kg body weight.

Cytotoxicity, as evidenced by increased DNA synthesis in both the liver and kidney of chloroform-treated mice, showed an increasing dose-response relationship at 60 and 240 mg chloroform/kg body weight, but it was normal at a nontumorigenic dose of 15 mg/kg (Table 4). Concomitant with the increase in DNA synthesis, histopathological alterations were observed at the higher dose levels. On the other hand, NDMA, at 3 mg/kg body weight, showed no propensity towards increasing DNA synthesis or histopathological alterations as evidence of cytotoxicity.

In summary of the chloroform data, DNA alkylation in the liver following a tumorigenic dose level of chloroform was a maximum of three alkylations per 10^6 nucleotides. DNA repair was not detectable. A review of the literature has also shown that chloroform is not mutagenic in a mammalian cell and several bacterial mutagenesis assay systems (Kirkland, Smith & Van Abbé, 1981; Reitz *et al.*, 1980). Evidence of chloroform's cytotoxicity has come from studies using mice dosed with a tumorigenic level of 240 mg/kg body weight. DNA synthesis was observed to be increased 14-fold in the liver and 25-fold in the kidneys of these animals, and this was associated with histopathological observations of toxicity. It appears that the genetic potential of chloroform is nil to very little, while the component of recurrent cytotoxicity is very significant. Thus, as with perchloroethylene, it appears that chloroform causes tumours in mice *via* a cytotoxic mechanism rather than by a purely genetic mechanism.

The final example is that of 1,4-dioxane. When chronically administered to rats at a concentration of 0·75 to 1·8% in their drinking-water, 1,4-dioxane has been observed to cause an excess incidence of nasal and hepatocellular carcinomas (Argus, Arcos & Hoch-Ligeti, 1965; Hoch-Ligeti, Argus & Arcos, 1970; Kociba, McCollister, Park, Torkelson & Gehring, 1974). Ingestion of lower doses (0·1%) in drinking-water (Kociba *et al.* 1974) and inhalation of 1,4-dioxane (Torkelson, Leong, Kociba, Richter & Gehring,

Table 4. *Data on chloroform and N-nitrosodimethylamine (NDMA) genotoxicity and cytotoxicity in male CD-1 mice (Reitz, Quast, Stott, Watanabe & Gehring, 1980)*

Treatment	Hepatic DNA: Binding (alkylations/ 10^6 nucleotides)	Hepatic DNA: Repair*	DNA synthesis† in: Liver	DNA synthesis† in: Kidney	Histopathological changes‡ in: Liver	Histopathological changes‡ in: Kidney
Chloroform						
240 mg/kg	3	ND	× 14	× 25	+	+
60 mg/kg	—	—	× 2·2	× 8·2	−	+
15 mg/kg	—	—	ND	ND	−	−
NDMA:						
20 mg/kg	—	7·37	—	—	—	—
10 mg/kg	540	3·04	—	—	−	—
3 mg/kg	—	1·60	ND	—	−	—

ND = Not detected

*Ratio of treated control values. Values > 1·0 indicate an increase in DNA repair.

†Presented as multiples of control values as measured by [6-^{3}H]thymidine incorporation.

‡A treatment-related response was observed (+) or no treatment-related effect was observed (−).

Chloroform was administered as a single oral dose; NDMA was given as a single ip injection.

1974) have failed to produce excess tumours. At a tumorigenic dose level (1015 mg/kg/day or 1% in water), 1,4-dioxane was also observed to cause pronounced hepatic degenerative and regenerative changes (Kociba *et al.* 1974).

No genotoxicity has been observed in Sprague–Dawley rats dosed with a single oral carcinogenic dose level of 1,4-dioxane (1000 mg/kg); no increased DNA repair or hepatic DNA alkylation was detected. *In vitro* mutagenicity assays carried out following the methods of Ames, Durston, Yamasaki & Lee (1975) and using *S. typhimurium* strains TA98, 100, 1535, 1537 and 1538 also gave negative results. No DNA repair was detected in an *in vitro* primary rat hepatocyte RNA repair assay following the method of Williams (1977). Yet, as shown in Table 5, repeated administration of a carcinogenic dose level of 1,4-dioxane (1000 mg/kg body weight/day) was observed to be cytotoxic to rat hepatic tissue as evidenced by a 1·5-fold increase in hepatic DNA synthesis and abnormal histopathology relative to the controls. No cytotoxic changes were observed in rats dosed with a non-tumorigenic level of 1,4-dioxane (10 mg/kg body weight/day).

The lack of genotoxicity and the observed cytotoxicity of 1,4-dioxane at tumorigenic dose levels indicates that as with perchloroethylene and chloroform in the mouse, 1,4-dioxane may cause tumours in the rat *via* an epigenetic-cytotoxic mechanism of action. However, the pronounced hepatocellular hypertrophic response observed in rats exposed to a high dose level of 1,4-dioxane (Table 5) and the observation that 1,4-dioxane induces hepatic drug-metabolizing enzyme systems in the rat (this laboratory) also suggest another epigenetic mechanism of tumorigenic action characteristic of metabolic inducing agents such as phenobarbital. It has been suggested that this mechanism of action may be a result of degranulation of rough endoplasmic reticulum resulting in alterations in protein synthesis and gene expression or by a hyperplasia-induced enhancement of the expression of pre-existing oncogenic factors (Tennekes, 1979; Wright, Akintowa & Wooder, 1979). Thus from the available data it appears that repeated exposure to high doses of 1,4-dioxane may cause tumours in rats *via* one or more epigenetic (mutational or nonmutational) mechanisms of action.

Conclusion

Obviously, much work remains to be done to fill in the gaps in our understanding of the mechanisms of chemical carcinogenesis. Future work to define critical sites on macromolecules (DNA, histones, nonhistone proteins) that may be important in the process of gene expression and cellular transformation will be of particular importance. However our understanding of the mechanisms of chemical carcinogenesis has reached a point whereby this knowledge may be used to generally classify carcinogens and in human risk assessment, even though the more definitive mechanistic details remain to be elucidated. The studies cited above, and others, have shown that all carcinogens do not act by the same mechanism. Some, which have genetic mechanisms of carcinogenicity, pose greater risks than others, which act by epigenetic mechanisms. Finally, it should be stressed that chemicals having an apparent cytotoxic mechanism of action are tumorigenic only at toxic dose levels, have reversible cytotoxicity, require multiple dosages, and appear to have realistic thresholds.

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Table 5. *Data on cytotoxicity of 1,4-dioxane in Sprague–Dawley rats*

Treatment	Dose (mg/kg/day)	Liver weight: body weight*	Hepatic DNA synthesis*	Hepatic histopathology†
Exposure *via* drinking-water for 11 wk	10	× 1·00	× 1·23	–
	1000	× 1·12	× 1·50	+

*Expressed as multiples of the control value.

†Treatment-related effects were observed (+) or no treatment-related effects were observed (–).

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LIVER CARCINOGENESIS: THE ROLE FOR SOME CHEMICALS OF AN EPIGENETIC MECHANISM OF LIVER-TUMOUR PROMOTION INVOLVING MODIFICATION OF THE CELL MEMBRANE

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Summary—Chemicals that produce tumours exclusively or primarily in the liver of rodents following prolonged administration at high dose levels, that show no capacity to induce genetic damage, but that enhance the carcinogenic effect of previously administered genotoxic carcinogens are identified as epigenetic carcinogens of the promoter class. Recent findings suggest that these chemicals may affect the state or function of the cell membrane in such a way as to interfere with the transmission of regulatory factors from normal to spontaneously altered cells, thus releasing the latter for progressive neoplastic growth. This hypothesis is consistent with the dose-response characteristics of carcinogens of this type.

Introduction

Various drugs and chemicals produce tumours primarily or exclusively in the liver when they are administered for prolonged periods at high dose levels (Table 1). The properties of these substances differ considerably from those of the more thoroughly studied carcinogens described by Miller & Miller (1976) as giving rise to reactive electrophiles. The latter are sometimes effective with a single exposure, are often active at low doses, and generally produce tumours at multiple sites, usually after short or moderate latent periods. Partly because of such differences in the carcinogenic effects of diverse carcinogens, Weisburger & Williams (1980) have proposed the classification of two major categories of carcinogen, genotoxic and epigenetic.

Genotoxic carcinogens are defined as those that are capable of producing DNA damage through covalent reactions, and thus they correspond to carcinogens that act as electrophilic reactants. Epigenetic carcinogens are defined as those that do not damage DNA but act by indirect mechanisms. The genotoxic capacity of carcinogens can be evaluated by determining chemical modification of DNA or by short-term testing for genetic damage. Substantial evidence from short-term tests for genetic damage carried out on the hepatocarcinogenic chemicals listed in Table 1 indicates a lack of genotoxic action. Therefore, we have suggested that such chemicals are epigenetic carcinogens, probably of the promoter class (Williams, 1979 & 1980a).

It is consistent with this concept that several of these carcinogens, such as phenobarbital and DDT (Peraino, Fry & Grube, 1978), have been shown to enhance the carcinogenic effect of previously administered genotoxic carcinogens under conditions in which the proposed epigenetic carcinogens are not carcinogenic by themselves. This effect in sequential administration is usually interpreted as evidence of promoting activity, although in the absence of other information it could equally well represent a summation of multiple genotoxic effects (G. M. Williams, S. Katayama & T. Ohmori, unpublished work 1981). The process by which epigenetic hepatocarcinogens of the promoter class operate is being clarified by recent advances in the understanding of the mechanisms of liver-tumour promotion.

Inhibition of intercellular communication as a mechanism of tumour promotion

A tumour promoter is an agent that permits tumour formation by altered cells that would otherwise remain dormant. Although tumour promoters are usually identified by their enhancement of the yield of tumours resulting from a previously administered carcinogen, called an 'initiating' agent in the terminology adopted for two-stage carcinogenesis (Berenblum, 1974), they can also be conceived as promoting tumour formation by cells altered through

Table 1. *Chemicals that are hepatocarcinogenic after prolonged exposure*

Test material	Degree of hepatocarcinogenicity*	
	In mouse	In rat
Phenobarbital	++	+/−
Clofibrate	ND	+
Nafenopin	++	+
DDT	+	+
Dieldrin	++	−
Lindane	++	+/−
Mirex	++	+
PCB	++	+

*Evaluation of hepatocarcinogenic findings after long-term administration: +, active hepatocarcinogen; ++, more active than +; −, inactive; +/−, studies mostly negative; ND, not determined.

effects other than induced initiation, such as inherited genetic abnormalities or spontaneous mutation.

Two likely mechanisms (Fig. 1) have been postulated to underlie the process of promotion: (1) promoters could complete the conversion of partially transformed cells to fully neoplastic cells, which would then be capable of progressive growth into tumours (scheme A) or (2) promoters could act on neoplastic cells to enable them to proliferate into overt neoplasms (scheme B), a development that, without promotion, would be very limited.

The first hypothesis, that promoters complete or somehow make permanent neoplastic conversion, has been supported by reports of promoter effects on cell differentiation (Diamond, O'Brien & Rovera, 1978; O'Brien, 1976; Yamasaki, Fibach, Nudel, Weinstein, Rifkind & Marks, 1977) and on genetic organization (Kinsella & Radman, 1978). Demonstration of such effects, however, has been limited so far to the skin-tumour promoters, phorbol esters and teleocidin B (Fujiki, Mori, Nakayasu, Terada & Sugimura, 1979). These agents function at very low doses and have almost hormone-like characteristics. In fact, Weinstein, Nigler, Fisher, Sisskin & Pietropaolo (1978) have suggested that this class of promoters may act by usurping the action of endogenous hormone or growth-regulating substances. Alternatively, Boutwell (1978) and his associates have proposed that skin-tumour promoters induce the tumour phenotype in all epidermal cells and this phenotype cannot be reversed in initiated cells. These studies on the mechanism of action and promoting effect of phorbol esters have so far been restricted to skin and cell cultures.

The second possible means by which promoters may function, that is by assisting already neoplastic cells to form tumours, corresponds to the original concept of Berenblum (1974) and is more compatible with the process of promotion in the liver and possibly in other visceral organs. Although some liver-tumour promoters induce enzyme activities, reflecting a modulation of gene expression, they do not produce the effects on differentiation and chromosomes reported for phorbol esters. Thus, no substantial evidence exists for any action that might be construed as completing the process of neoplastic conversion at the level of genetic organization. Much interest has focused on the effects of promoters on cell membranes (Boyland, 1980; Driedger & Blumberg, 1977; Rohrschneider, O'Brien & Boutwell, 1972; Sivak, 1972), and recently, through the work of Yotti, Chang & Trosko (1979) and Murray & Fitzgerald (1979), a specific effect of promoters has been delineated which could augment the growth of restrained neoplastic cells into tumours. These groups have described the ability of tumour promoters to inhibit intercellular communication in fibroblast-cell cultures and we have confirmed this action in liver-cell cultures for the liver-tumour promoters, phenobarbital (Williams, 1980a) and DDT (Williams, Telang & Tong, 1981).

Cell-to-cell exchange of molecules occurs through membrane organelles known as gap junctions (Flagg-Newton, 1980; Pitts, 1980; Revel, Yancey, Meyer & Nicholson, 1980). This process can be measured in cell culture as the phenomenon of metabolic co-operation (cross feeding), in which a lethal metabolite generated from a precursor by one cell type is transferred to a mutant that cannot produce it; as a result, the mutant, which otherwise would be resistant to the effects of addition of the precursor, is passively killed. For example, in mixed liver cultures, the lethal mononucleotide of 6-thioguanine (TG) is transferred from freshly isolated normal hepatocytes to 6-thioguanine-resistant (TG^r) mutant adult rat-liver epithelial cells. The hepatocytes possess the purine salvage pathway enzyme, hypoxanthine–guanine phosphoribosyl transferase (HGPRT), which is required to metabolize TG to the toxic metabolite. The TG^r mutant liver-cell strain, which is selected by its resistance to TG (Tong & Williams, 1980), lacks HGPRT and is not affected by TG unless the metabolite is transferred to it through metabolic co-operation. In this system, the degree of metabolic co-operation is measured by the reduction in colony formation by TG^r cells co-cultivated with freshly isolated hepatocytes in primary culture. As Trosko and associates have described in their system (Trosko, Dawson, Yotti & Chang, 1980; Yotti *et al.* 1979), inhibition of metabolic co-operation can be measured by restoration of colony formation by the mutants. The use of isolated hepatocytes in this system is advantageous because the cells presumably retain elements of *in vivo* cell characteristics and, from a practical point of view, they are incapable of colony formation in primary culture and thus do not interfere with the determination of colony formation by the target TG^r cells. The inhibition by DDT of metabolic co-operation between hepatocytes and adult rat-liver epithelial-TG^r cells is shown in Table 2.

The inhibition of intercellular communication between liver cells demonstrated in cell culture could be the basis for *in vivo* promotion of liver tumours. In all tissues, cell proliferation is a precisely controlled process in which the production of new cells balances those lost through differentiation or death. The regu-

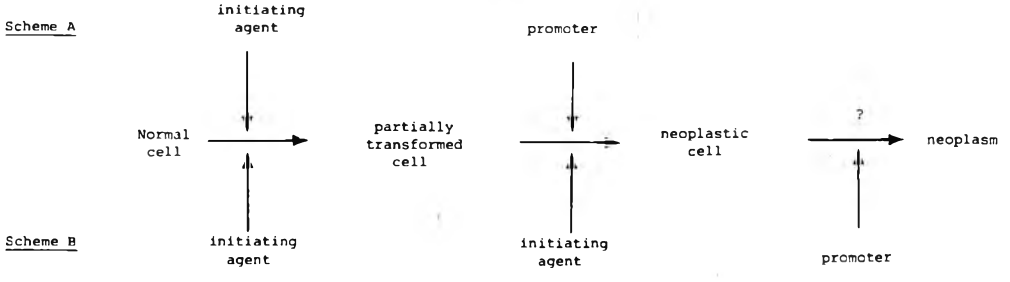

Fig. 1. Schemes for the action of tumour promoters.

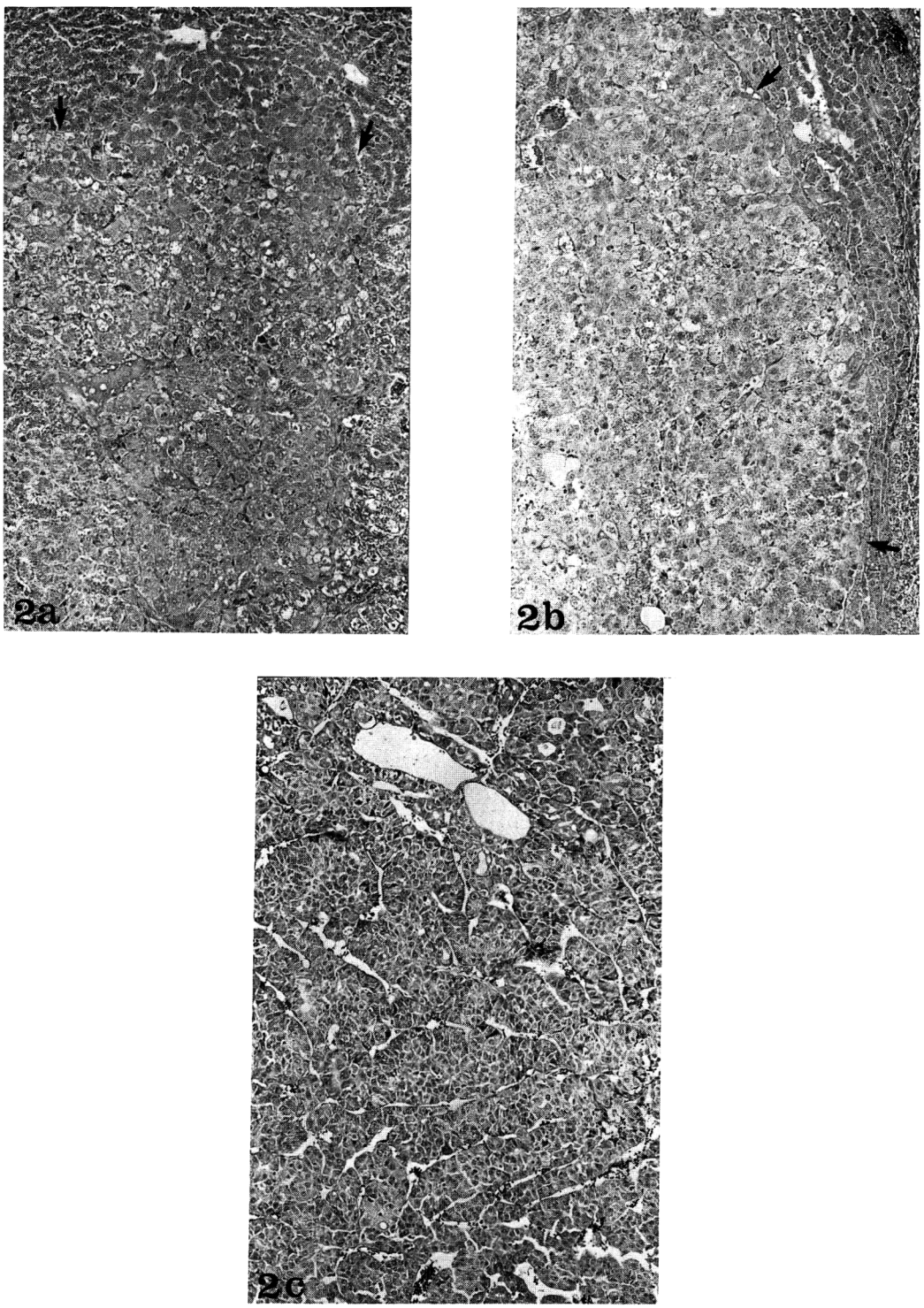

Fig. 2. (a) Altered focus induced by 2-acetylaminofluorene in rat liver; the enlarged cells of the altered focus (arrowed) are morphologically distinct from normal hepatocytes (top) but are in continuity with the hepatic plates. (b) Neoplastic nodule induced by 2-acetylaminofluorene in rat liver; the enlarged cells of the neoplastic nodule (arrowed) are sharply demarcated from the adjacent normal hepatocytes (right). (c) Hepatocellular carcinoma induced by 2-acetylaminofluorene in rat liver; the malignant neoplasm is composed of broad trabeculae of highly atypical neoplastic cells. Haematoxylin and eosin $\times 200$

Table 2. *Inhibition of metabolic co-operation between hepatocytes (HPC) and an adult rat-liver epithelial strain (ARL 14-TG^r) by a liver-tumour promoter, DDT**

Culture conditions	TG^r colonies/flask†	
	Without HPC	With HPC
ARL 14-TG^r	126 ± 7	—
+TG	110 ± 3	63 ± 10
+TG + DDT 10^{-7}	103 ± 9	86 ± 4
+TG + DDT 10^{-6}	101 ± 13	112 ± 10
+TG + DDT 10^{-5}	105 ± 11	117 ± 6

TG = 6-Thioguanine

*Data from Williams *et al.* (1981).

†Values are averages for three flasks of 500 TG^r cells cultured alone or co-cultured with 0·75 × 10^6 HPC.

lation of this process is incompletely understood, but could involve cell-to-cell transfer of factors that regulate growth, including chalones and other substances that induce differentiation and, thereby, loss of proliferative capability. Several lines of evidence indicate that growth regulation is exerted on neoplastic cells. Most significantly, an interval of up to 1 yr can be allowed between initiation and the beginning of promotion with essentially the same incidence of tumours occurring as when promotion immediately follows initiation (Van Duuren, Sivak, Katz, Seidman & Melchionne, 1975). It seems certain that during the prolonged interval between initiation and promotion, neoplastic cells undergo turnover in the tissue along with normal cells and are kept under control by the regulatory factors that operate on normal cells. Nettesheim, Klein-Szanto, Marchok, Steele, Terzaghi & Topping (1981) have strengthened this concept with findings showing that the number of altered cells produced by exposure of the respiratory tract to a carcinogen exceeds considerably the number that will become manifest as neoplasms. Other examples of the control of neoplastic cells are the induction of differentiation in (1) cells receiving a transplanted nucleus from a neoplastic cell (King & DiBerardino, 1965), (2) neoplastic cells associated with normal embryonic cells *in utero* (Mintz & Illmensee, 1975; Papaioannou, McBurney & Gardner, 1975) and (3) neoplastic cells exposed to embryo extracts (DeCosse, Gossens, Kuzma & Unsworth, 1975; Ellison, Ambrose & Easty, 1969). If the control of dormant neoplastic cells can be mediated by intercellular communication, as seems likely, then inhibition of communication could release the neoplastic cells for the progressive growth that results in the formation of neoplasms. Thus, the inhibition of intercellular communication could be the basis for tumour promotion. It is noteworthy that this effect, although not perfectly correlated with promoting activity (Umeda, Noda & Ono, 1980), is the only property that has been demonstrated for a variety of tumour promoters (Fitzgerald & Murray, 1980; Trosko *et al.* 1980; Umeda *et al.* 1980; Williams, 1980a; Williams *et al.* 1981; Yotti *et al.* 1979).

The inhibition of intercellular communication by promoters can conceivably be produced in several ways. Because DDT is lipophilic, it may accumulate in the lipid layer of the cell membrane and interfere directly with the function of gap junctions. The means of inhibition by phenobarbital, which is not lipophilic, may be more complex. We have shown that phenobarbital affects the activity of several membrane-associated enzymes in mouse-liver tumours (Williams, Ohmori, Katayama & Rice, 1980) and recently we have found that it affects membrane functions in cultured liver cells (Shimada, Kreiser & Williams, 1981). It seems likely, therefore, that the action of phenobarbital is mediated by its effect on gene expression, giving rise to alterations in the composition of the cell membrane. Other effects by surface-active substances can also be visualized. In addition to these biochemical mechanisms, physical separation of cells could operate *in vivo* to interrupt communication between cells.

A hypothetical sequence for neoplastic development in rodent liver

We have previously proposed a sequence of cellular events in the development of experimental hepatocellular carcinoma (Williams, 1980b). The essential features of this proposal were that genetic alterations in hepatocytes rendered them capable of forming altered foci (Fig. 2a) and that altered foci could progress to the formation of neoplastic nodules (Fig. 2b), a type of possibly benign neoplasm, or to the formation of hepatocellular carcinomas (Fig. 2c).

The foregoing considerations now permit the proposal of a more detailed hypothesis of neoplastic development in rodent liver (Fig. 3). As a function of the extent of genetic alteration produced by a genotoxic carcinogen, a spectrum of changes may occur in liver cells, including alterations insufficient to direct the neoplastic state, alterations sufficient to direct the neoplastic state but insufficient for abnormal growth, alterations sufficient to direct the neoplastic state and sufficient for only limited abnormal growth and alterations sufficient both to direct the neoplastic state and to permit progressive growth into neoplasms.

In the first condition (i.e. alterations insufficient to direct the neoplastic state), the altered cell could remain dormant as a partially transformed cell, designated (a) in Fig. 3. Additional genotoxic alterations would be required before conversion of the altered cell to a neoplastic cell (b). Such additional alterations could be the basis for the cumulative effect of subsequent carcinogen exposures. As a result of the genetic alterations or with the assistance of a promoter, the partially transformed cell could proliferate to

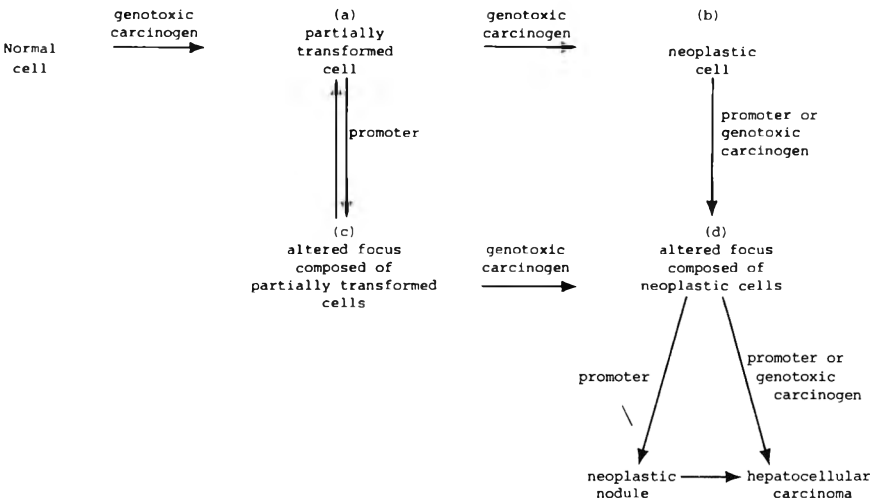

Fig. 3. Hypothetical sequence for neoplastic development in the rodent liver.

form an altered focus (c), which would be highly susceptible to phenotypic reversion but which, without additional genetic alterations, would not progress to form neoplastic lesions.

In the second and third conditions, the cell is completely converted to a neoplastic cell through genetic alterations but is limited in its growth capability. In the second condition, the neoplastic cell remains inapparent (b), whereas in the third it proliferates to form an altered focus (d). A major factor in restricting the growth of the neoplastic cells at these stages could be the imposition of differentiation on them by intercellular transmission of regulatory factors. Thus, in these stages, chemicals that inhibit intercellular communication can exert a promoting action by releasing the neoplastic cells from regulation and liberating them for progressive growth according to their altered genetic capability. Continuous maintenance of interrupted intercellular communication would be required in order for these dormant neoplastic cells to progress to form tumours. Once a sufficient mass of neoplastic cells is achieved, the majority of neoplastic cells would be effectively isolated from interaction with surrounding normal cells and thus would no longer be dependent upon the promoter.

In the fourth condition, the genetic alterations in the cell are sufficient for neoplastic conversion and cell-membrane alterations that decrease its permeability to normal tissue constituents regulating growth and differentiation. The progeny of such a cell would be able to grow in a progressive manner without assistance from chemicals that inhibit intercellular communication.

Role of promotion in the carcinogenicity of epigenetic hepatocarcinogens

As indicated earlier, some chemicals produce tumours exclusively or primarily in the liver in rodents and show no evidence of genotoxic effects. Several of these have been shown to have an enhancing effect on the carcinogenicity of a previously administered genotoxic carcinogen. Thus, they appear to be epigenetic carcinogens of the promoter class. We have previously pointed out (Williams, 1979 & 1980b) that the hepatocarcinogenicity of these chemicals may be due to their promoting action on genetically altered cells in the livers of rodents with a spontaneous incidence of liver tumours. In the light of the recent findings described here, it may be visualized that these chemicals interfere with the transmission of regulatory factors from normal cells to spontaneously altered cells, thereby releasing the latter for progressive growth as neoplasms.

This concept would explain some of the dose responses characteristic of carcinogens of this type. Such carcinogens would have to be administered in a sufficiently high dose to alter the cell membrane extensively, either through accumulation in the lipid layer as with DDT or through alteration of the membrane composition as with phenobarbital, in order to inhibit intercellular exchange at the many sites of communication between cells. In addition, such chemicals would have to be administered for a period long enough to enable the altered cells either to achieve sufficient mass to insulate themselves from regulatory signals transmitted by the normal tissue or to acquire during proliferation additional abnormalities that would enable them to become resistant to intercellular communication.

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REVIEW OF THE HEPATIC RESPONSE TO HYPOLIPIDAEMIC DRUGS IN RODENTS AND ASSESSMENT OF ITS TOXICOLOGICAL SIGNIFICANCE TO MAN

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Summary—Dietary administration of various chemically-unrelated hypolipidaemic (*viz.* hypotriglyceridaemic) drugs can induce liver carcinomas in rats or mice. These drugs, in common with other compounds exhibiting hypolipidaemic activity (e.g. di-(2-ethylhexyl) phthalate), produce in rodents an initial hepatic response characterized by the presence of (1) liver enlargement unaccompanied by frank histological liver damage, (2) proliferation of the smooth endoplasmic reticulum and (3) a striking increase in peroxisome numbers. Hypolipidaemic drugs exert differential effects on the activities of hepatic peroxisomal enzymes. Massive increases in the activities of enzymes involved in the β-oxidation of fatty acids and of carnitine acetyltransferase overshadow the modest increases, if any, in the activities of catalase and certain oxidase enzymes. It is suggested that the bioavailability of catalase in the liver peroxisomes of rodents treated with hypolipidaemic drugs is insufficient to cope with the detoxication of injurious H_2O_2 concentrations resulting from the considerably enhanced activity of H_2O_2-generating enzymes. Liver cells may thus be exposed to the cytotoxic or DNA-damaging potential of H_2O_2 as a result of the breakdown in homeostasis, and such exposure could lead to the subsequent development of neoplasia in the rodent liver. Evidence is accumulating that in man (as well as in monkeys) hypolipidaemic drugs do not elicit the peroxisome proliferative response that is associated with the disposal of hepatic lipids in rodents. Consequently it would not be justified to extrapolate the findings of rodent hepatocarcinogenesis to humans. Because of differences in response between the rodent liver and human liver towards the action of certain microsomal-enzyme inducers (phenobarbital, dieldrin, DDT, Ponceau MX and safrole), a similar conclusion has been reached—that the hepatocarcinogenicity of these compounds demonstrable in the rodent is not relevant to man. Moreover microsomal-enzyme inducers share remarkably close parallels with hypolipidaemic drugs in respect of the initial hepatic reaction, a lack of mutagenic potential/DNA interaction and the nature of the carcinogenic response. From the available data it is concluded that the hepatic peroxisome response is an important aetiological factor in liver cancer induced by hypolipidaemic drugs in rodents. The absence of a peroxisomal response in man (and subhuman primates) coupled with important differences in the way rats and humans handle lipoprotein remnants bound to liver cells strongly indicates that chronic administration of hypolipidaemic drugs is unlikely to present a liver-cancer risk in man.

Introduction

Various hypolipidaemic drugs/agents (e.g. clofibrate, fenofibrate, nafenopin, Wy-14,643 and tibric acid) induce striking changes in the liver of rats and mice. The initial hepatic response in rodents is characterized by (1) hepatomegaly, (2) proliferation of the smooth endoplasmic reticulum (SER) and (3) a marked increase in the number of peroxisomes and changes in the peroxisomal structure and enzyme composition (Barnard, Molello, Caldwell & LeBeau, 1980; Blane & Pinaroli, 1980; Hess, Stäubli & Riess, 1965; Moody & Reddy, 1978a; Reddy & Krishnakantha, 1975; Svoboda, Grady & Azarnoff, 1966). However, of much greater concern has been the induction of liver carcinomas in rats or mice by various hypolipidaemic drugs (Reddy, Azarnoff & Hignite, 1980; Reddy & Qureshi, 1979; Reddy & Rao, 1977a,b; Reddy, Rao, Azarnoff & Sell, 1979; Reddy, Rao & Moody, 1976a; Svoboda & Azarnoff, 1979), as well as by the hypolipidaemic phthalate esters (National Cancer Institute, 1980 & 1981).

Because the compounds in question are not all chemically related to one another, it has been suggested that hypolipidaemic agents that are capable of eliciting a persistent hepatic peroxisome proliferation and hepatomegaly are also capable of inducing liver

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tumours and thus represent a novel class of chemical carcinogens (Reddy *et al.* 1980). However, studies in monkeys (Blane & Pinaroli, 1980; Platt & Thorp, 1966; Svoboda *et al.* 1967) and in man (Blane & Pinaroli, 1980; Hanefeld, Kemmer, Leonhardt & Jaross, 1977; Hanefeld, Kemmer, Leonhardt, Kunze, Jaross & Haller, 1980; Schwandt, Klinge & Immich, 1978) have failed to provide evidence of the initial hepatic response that is so prominent in rats and mice. This has led to doubts as to the relevance of the rodent carcinogenicity findings to man, especially as it is conceivable that the initial hepatic response in rodents is in some way associated with the manifestation of liver tumours.

Studies on other compounds such as Ponceau MX and safrole have revealed biochemical and histochemical changes in the enlarged rat liver which are indicative of hepatotoxicity, and are deemed to assume importance in liver-tumour development in this species (Crampton, Gray, Grasso & Parke, 1977a,b; Grasso & Gray, 1977).

In the case of hypolipidaemic compounds, attention has been focused particularly on the initial hepatic response and to some extent on the carcinogenic end points. But scant regard has been paid to the sequential biochemical and pathological changes that take place in the liver in the intervening period and that could throw light on the mechanism by which hypolipidaemic agents exert their carcinogenic effect in rodents. It would appear, however, that this mechanism differs in one critical respect from that prevailing for the majority of true classical hepatocarcinogens (e.g. aflatoxin, nitrosamines), in that hypolipidaemic drugs do not interact with or cause damage to cellular DNA (Blane & Pinaroli, 1980; von Däniken, Lutz & Zbinden, 1981; Warren, Simmon & Reddy, 1980).

In an attempt to put these drug-induced phenomena in rodents into perspective, an overview is presented of the inter-relationships between liver enlargement and attendant liver changes encountered at the biochemical, histochemical, histological and ultrastructural levels mainly for hypolipidaemic agents but also for other compounds of comparative importance. An attempt has also been made to shed light on the significance of peroxisome proliferation in the hepatotoxic response and a pathway of events leading to tumour formation has been proposed. Above all the available data have been critically assessed to ascertain the extent to which the drug-induced phenomena seen in the rodent liver are relevant to man.

Hepatic response to hypolipidaemic drugs/agents

General

The liver is the primary site of toxic action of hypolipidaemic agents in rodents. The initial lesion consists of liver enlargement, elevated serum-transaminase levels, minor histological changes in the hepatocytes, proliferation of the SER and a striking increase in peroxisome numbers, accompanied by alterations in the peroxisomal structure and peroxisomal enzyme activities. These changes may ultimately lead to the formation of liver nodules and tumours in rodents. (Peroxisome proliferation and liver-tumour induction are discussed at length in subsequent sections.)

This overall response is characteristic of triglyceride-lowering agents (e.g. clofibrate) but is absent with the cholesterol-lowering agent, probucol, which exerts no effect on serum-triglyceride levels (Barnard *et al.* 1980). The difference in the hepatic response between hypotriglyceridaemic and hypocholesterolaemic drugs is also evident at the biochemical level. Thus hepatic activities of catalase, lactic dehydrogenase, glucose 6-phosphatase and glutamic–pyruvic transaminase (GPT) and hepatic glycogen content were unaffected by probucol treatment of rats (0.25% in the diet for 2 wk) but these parameters, with the exception of glucose 6-phosphatase, were elevated by similar treatment with clofibrate (Lepetit Pharmaceuticals Ltd, 1980).

In addition to the difference in hepatic response between these two types of hypolipidaemic drug, it is important to draw attention at the outset to species differences in the hepatic response to hypolipidaemic (*viz.* hypotriglyceridaemic) drugs/agents between rodents on the one hand and monkeys and man on the other.

Acute, short-term and subchronic studies in rodents and dogs

Rats. Studies involving seven daily oral doses of 3–300 mg fenofibrate/kg or of 20–600 mg clofibrate/kg to male Wistar rats revealed dose-dependent liver enlargement for each compound, the effect with fenofibrate being seen only above 30 mg/kg. Hepatomegaly was unaccompanied by histological change in the liver but was accompanied by an increase in the numbers and size of peroxisomes (G. F. Blane, unpublished report 1979).

A further study in male Wistar rats given daily oral doses of fenofibrate (100 mg/kg) or clofibrate (200 mg/kg) for 1 wk revealed that liver enlargement was accompanied by depressed activity of the microsomal enzymes, coumarin hydroxylase and aminopyrine demethylase, but there was no effect on microsomal glucose 6-phosphatase, phospholipid or protein (G. F. Blane, unpublished report 1979).

In a comparative study of Egyt 1299 and clofibrate given to male rats in ten daily oral doses of 300 and 30 mg/kg, respectively, liver enlargement was accompanied by proliferation of the SER and rough endoplasmic reticulum (RER), by marked increases in peroxisomes and by increases in microsomal aminopyrine-demethylase activity and cytochrome *P*-450 content (Elek & Jambor, 1978). Egyt 1299 increased microsomal protein whilst clofibrate increased hexobarbital oxidase.

In nafenopin-treated rats (0·125 or 0·25% in the diet for 4 wk), the liver cells showed marked proliferation of the SER, isolated channels of RER, normal mitochondria and no increase in lysosomes or in lipid droplets, but many hepatocyte nucleoli were hypertrophied (Reddy, Azarnoff, Svoboda & Prasad, 1974).

Di-(2-ethylhexyl) phthalate given orally at 2 g/kg/day to male Wistar rats for 4–21 days induced hepatomegaly, accompanied by dilatation of the SER and RER, a peroxisomal increase and mitochondrial swelling (with shortening of the cristae), but no overt histological changes were seen (Lake, Gangolli, Grasso & Lloyd, 1975). The initial increase in alcohol-dehydrogenase activity and in microsomal protein

and cytochrome *P*-450 was followed by a partial reversal at 21 days. The biphasic response resembled that of dieldrin and safrole. Reductions of microsomal glucose 6-phosphatase and aniline 4-hydroxylase and of mitochondrial succinic-dehydrogenase activity throughout the exposure period were demonstrated both biochemically and histochemically.

In a 28-day study in progress in rats, fenofibrate was administered in the diet to provide daily intakes of 0, 13, 60 or 200 mg/kg; another group received clofibrate at 400 mg/kg. Preliminary results indicate the occurrence of liver enlargement in all test groups, becoming more marked with increasing fenofibrate dose (S. C. Price, R. H. Hinton, J. W. Bridges & P. Grasso, unpublished observations 1981). Histologically, a decrease in glycogen deposits and periportal accumulation of lipid droplets in the liver were observed with both compounds. These findings were confirmed on ultrastructural examination, which also demonstrated peroxisome proliferation (absent with 13 mg fenofibrate/kg) and SER proliferation. Biochemically, catalase activity in liver homogenates increased slightly with increasing fenofibrate dose, but peroxisomal catalase activity was reduced. Glucose 6-phosphatase was depressed at all levels in a dose-related manner. The cytochrome *P*-450 content showed a transient increase at the two highest levels of fenofibrate, returning to normal at 60 mg/kg and to below-normal levels at 200 mg/kg. Ethoxycoumarin de-ethylase activity was hardly affected at the two lowest levels but increased transiently and then fell to below the basal level at the 200-mg/kg level. A marked fall in microsomal protein was also seen with 200 mg fenofibrate/kg. In general the biochemical effects seen with 400 mg clofibrate/kg were similar to those elicited with 60 mg fenofibrate/kg.

In a 3-month study in male Sprague–Dawley rats, fenofibrate was given in daily oral doses of 50–1000 mg/kg (Blane & Pinaroli, 1980). Liver enlargement occurred at all dose levels, progressively increasing by 27–74% of control values as doses increased from 50 to 1000 mg/kg/day; levels of serum glutamic–oxalacetic and serum glutamic–pyruvic transaminases (SGOT and SGPT) increased transiently at the two highest levels of 500 and 1000 mg/kg/day but no histological changes were evident in the liver at any dose level.

The similar effects on liver weight and on liver histology and ultrastructure that are manifest with the hypolipidaemic agents clofibrate and fenofibrate are shown in Table 1, summarizing the findings of Barnard *et al.* (1980). In this study, Sprague–Dawley rats received diets providing daily intakes of 250 mg clofibrate/kg for 91 days or of 100 mg fenofibrate/kg for 28 days. In striking contrast, probucol given similarly to rats at 500 mg/kg for 91 days failed to induce either liver enlargement, gross or histological changes of the liver, any increase or changes in liver peroxisomes or any ultrastructural changes in other cytoplasmic organelles, except for a possible increase in the SER.

Mice. Histological examination of the markedly enlarged livers of wild-type (Cs^a strain) mice fed 0·1% nafenopin for up to 56 wk, at which time the last of the animals died, showed large polygonal parenchymal cells with abundant eosinophilic granular cytoplasm, resembling megalocytes (Reddy *et al.* 1976a). Liver biopsies obtained at 60–70 wk from acatalasemic mice (Cs^b strain with unstable catalase gene) fed nafenopin (0·1% in the diet for 12 months; 0·05% thereafter) revealed small foci of neoplastic nodules composed of cells with hyperbasophilic cytoplasm (Reddy *et al.* 1976a).

Dogs. Clofibrate (35–45 mg/kg given orally on alternate days for 3 months) resulted in hepatomegaly in female but not in male beagles (Platt & Thorp, 1966). When fenofibrate was administered in the diet to eight dogs, providing intakes of 25–100 mg/kg/day for 7 to 24 months, alkaline phosphatase was raised in males given 50 or 100 mg/kg and in females on 25 mg/kg but the SGPT response was variable; no liver enlargment or histological changes in the liver were reported (Blane & Pinaroli, 1980).

Studies in monkeys (1 wk–2 yr)

Rhesus monkeys given clofibrate at 2% in the diet for 3 months showed a slight increase in liver weight, but no effect on liver weight was seen following 0.5–2% in the diet for 2 yr (Platt & Thorp, 1966). In the 2-yr study, the clofibrate preparation contained 2·2% (w/v) androsterone but its presence was not con-

Table 1. *Effect of probucol, clofibrate and fenofibrate on rat liver**

Liver parameter	Response in rats† treated with:		
	Probucol	Clofibrate	Fenofibrate
Liver enlargement	–	+	+
Proliferation and enlargement of peroxisomes	–	+	+
Hepatocytomegaly	–	+	+
Cytoplasmic granularity	–	+	+
Increased SER	slight	+	+
Decreased, disarrayed RER	–	+	+
Ribosomal detachment	–	+	+
Glycogen depletion	–	+	+
Mitochondria—pleomorphic profiles	–	+	+

– = No effect + = Effect SER = Smooth endoplasmic reticulum
RER = Rough endoplasmic reticulum

*Data from Barnard *et al.* (1980).

†Sprague–Dawley rats received diets providing daily intakes of 250 mg clofibrate/kg for 91 days, 100 mg fenofibrate/kg for 28 days or 500 mg probucol/kg for 91 days.

sidered to alter the liver-weight response. No evidence of peroxisomal proliferation was obtained in one male squirrel monkey given daily oral doses of 75 mg clofibrate/kg for 22 days (Svoboda *et al.* 1967).

Four groups, each of 8–10 male and 8–10 female Rhesus monkeys, received orally 0, 12, 50 or 200 mg fenofibrate/kg/day for 1 yr. No effect was exerted on serum transaminases or alkaline phosphatase, on liver weight or on liver histology at any dose level (Blane & Pinaroli, 1980). In particular there was no increase in the number of liver peroxisomes in these fenofibrate-treated monkeys or in monkeys treated with 200 mg clofibrate/kg/day for 1 yr.

Studies in man

Histological examination of the biopsied livers of 40 hyperlipoproteinaemic patients before and after 3 months of clofibrate treatment (1·5 g/day in 27 patients; 0·5 g/day in 13 patients) revealed no drug-induced changes apart from some tendency to decreased fatty infiltration (Schwandt *et al.* 1978).

In other studies (Hanefeld *et al.* 1977 & 1980), no cellular damage was seen in liver-biopsy specimens of clofibrate-treated patients. Decreases in liver glycogen and manganese and an increase in mitochondria and SER were reported.

Elevated levels of SGOT and SGPT occurred in two of 17 patients treated with nafenopin—the effect was reversible (Dujovne, Weiss & Bianchine, 1971). Elevated transaminase activity occurred sporadically in fenofibrate-treated patients (Blane & Pinaroli, 1980). Transient increases of serum urea and/or creatinine were observed during fenofibrate treatment of hyperlipidaemic patients (Daubresse, 1980).

Liver biopsies revealed steatosis in nine of 18 hyperlipoproteinaemic controls compared with one of eight patients on fenofibrate (300 mg/day for 9 months) and in three of five patients on other hypolipidaemic drugs. A few peroxisomes were present in one fenofibrate patient and one control patient (Blane & Pinaroli, 1980).

Blane & Pinaroli (1980) refer to an unpublished study by N. A. Edmondson, D. E. Prentice & W. Schwartzkopff (1981) in which liver-biopsy specimens were taken from 28 patients given fenofibrate for periods of 2 months to 7 yr (mean 2·0 yr) and from 20 control untreated hyperlipidaemic subjects. No difference was found in peroxisome numbers between the two groups. Hanefeld *et al.* (1980) found no evidence of peroxisome proliferation in clofibrate-treated patients.

Epidemiological studies have failed to reveal any excess liver-cancer risk in clofibrate-treated patients (IARC Working Group, 1980).

Nature of hepatomegaly induced by hypolipidaemic agents in rodents

Liver enlargement and peroxisome proliferation are frequently observed after administration of various hypolipidaemic compounds (Table 2) and both are sustained over 25 months of nafenopin treatment in rats (Reddy & Rao, 1977a). However, these two findings do not always run in parallel. For example, liver enlargement induced by clofibrate can occur in the absence of peroxisomal proliferation (Svoboda *et al.* 1967). Also 14 days after clofibrate treatment ceased in rats, liver weight had almost returned to normal but peroxisome proliferation still persisted (Hess *et al.* 1965; Svoboda *et al.* 1967). Moreover whilst actinomycin D does not prevent clofibrate-induced hepatomegaly it impairs clofibrate-induced peroxisome proliferation (Svoboda *et al.* 1967).

Differences have been claimed in the mechanism of liver enlargement induced by clofibrate and nafenopin (Beckett, Weiss, Stitzel & Cenedella, 1972). On the basis of relationships between increases in liver size and changes in hepatic DNA content (relative and total), it has been estimated that after treatment of mice for 1 wk with nafenopin (100 mg/kg/day), one third to one half of the liver enlargement is due to hyperplasia of the hepatocytes and the rest to hypertrophy. After 2 wk of such treatment, at least one half of the increased liver size is due to hyperplasia. The increase in liver size produced by clofibrate, which is less marked than that encountered with nafenopin, can be explained solely by increases in the size of the hepatocytes, i.e. hypertrophy (Beckett *et al.* 1972; Hess *et al.* 1965). With nafenopin, the liver content of RNA, of soluble cytosol protein and of microsomal protein increases in proportion to the hepatomegaly (Beckett *et al.* 1972).

Liver enlargement can frequently be associated with microsomal-enzyme induction (Golberg, 1966). However, neither clofibrate nor nafenopin treatment affected the specific activities of the microsomal enzymes hexobarbital oxidase and aniline *p*-hydroxylase in mice (Beckett *et al.* 1972), although clofibrate induced a slight elevation of aminopyrine demethylase (Platt & Cockrill, 1969). According to Beckett *et al.* (1972) these two hypolipidaemic drugs cannot be regarded as consistent stimulators of microsomal drug metabolism. The lack of a consistent effect on microsomal-enzyme induction by hypolipidaemic agents is also evidenced by the demonstration of enzyme induction with Egyt 1299 by Elek & Jambor (1978) and with fenofibrate and clofibrate by S. C. Price *et al.* (unpublished observations 1981) but not with clofibrate, fenofibrate and tibric acid in the study of Holloway & Orton (1980) or with di-(2-ethylhexyl) phthalate (Lake *et al.* 1975). Preliminary results obtained with fenofibrate and clofibrate indicate a biphasic response of microsomal-enzyme induction, the transient increase being followed either by a return to normality or to below basal levels (S. C. Price *et al.* unpublished observations 1981). This finding could account for the variable results so far obtained in studies using different doses and times of enzyme assay. G. F. Blane (unpublished report 1979) has also suggested that peroxisome proliferation rather than microsomal-enzyme induction is a contributory factor in liver enlargement.

In a comparative study of the hepatic effects of clofibrate and two other hypolipidaemic agents, 3,9-di-3-pyridyl-2,4,8,10-tetraoxaspiro-5,5-undecane (compound A) and 2-(4-dibenzofuranyloxy)-2-methylpropionic acid (compound B), hepatic enlargement unaccompanied by histological change in the liver was seen with each of the three compounds in rats given daily oral doses of 250–300 mg/kg for 10 wk. Whilst peroxisome proliferation was observed with clofibrate and the more potent hypolipidaemic compound B but not with compound A, all three com-

Table 2. *Correlations between hepatic peroxisome proliferation, hypolipidaemic activity and hepatomegaly in rats or mice*

Compound	Hepatic peroxisome proliferation	Hypolipidaemic activity	Hepatomegaly*	References†
Triglyceride-lowering agents				
Fenofibrate	+	+	+	1, 2
Clofibrate	+	+	+	3, 4
Nafenopin	+	+	+	4, 5
Methyl clofenapate	+	+		4, 6
SaH-42-348	+	+	+	4, 7
S-8527	+	+		4
Egyt 1299	+	+	+	9
Wy-14,643	+	+	+	4
Tibric acid	+	+	+	4
RMI 14,514	+	+	+	14
Dimethrin	+	+		19
Di-(2-ethylhexyl) phthalate	+	+	+	10, 11
Di-(2-ethylhexyl) adipate	+	+	+	10
Di-(2-ethylhexyl) sebacate	+	+	+	10
2-Ethylhexyl alcohol	+	+	+	10
2-Ethylhexyl aldehyde	+	+	+	10
2-Ethylhexanoic acid	+	+	+	10
Acetylsalicylic acid	+	+		12, 13
3,9-Di-3-pyridyl-2,4,8,10-tetra-oxaspiro-5,5-undecane	−	+	+	15
2-(4-Dibenzofuranyloxy)-2-methylpropionic acid	+	+	+	15
Cholesterol-lowering agents				
Probucol	−	+	−	1, 8
20,25-Diazacholesterol	−	+		16
Benzmalacene	−	+		16
Benzyl-*N*-benzylcarbethoxy-hydroxamate	−	+		16
Dextran	−	+		16
Bovine catalase (caperase)	−	+		16
2,4-Dichlorophenoxyacetic acid	−	+		16
Chlorcyclizine	+	+	+	17
N-(Benzyloxy)-*N*-(3-phenylpropyl)-acetamide	+	+	+	17
1-Benzylimidazole	+	+	+	17
Boxidine	−	+	±	17
Haloperidol	−	+	±	17
Non-hypolipidaemic agents				
Hexyl alcohol	−	−	−	10
Hexanoic acid	−	−	−	10
Wy-14,643 analogues	−	−		18

+ = Effect − = No effect ± = Minimal effect

*Blank space denotes not examined/reported.

†References: (1) Barnard *et al.* (1980); (2) Blane & Pinaroli (1980); (3) Hess *et al.* (1965); (4) Reddy & Krishnakantha (1975); (5) Beckett *et al.* (1972); (6) Reddy *et al.* (1974); (7) Hartman, Bagdon, Van Ryzin & Tousimis (1970); (8) Lepetit Pharmaceuticals Ltd. (1980); (9) Elek & Jambor (1978); (10) Moody & Reddy (1978b); (11) Reddy *et al.* (1967b); (12) Hruban *et al.* (1966); (13) Ishii & Suga (1979); (14) Svoboda (1978); (15) Magnusson & Magnusson (1977); (16) Svoboda *et al.* (1967); (17) Hruban *et al.* (1974b); (18) Reddy *et al.* (1979); (19) Hruban, Gotoh, Slesers & Chou (1974a).

pounds induced proliferation of the SER. It was considered that peroxisome proliferation contributed more to hepatomegaly than did proliferation of the SER for clofibrate and compound B (Magnusson & Magnusson, 1977).

In summary, it can be said that peroxisome proliferation is very closely but not invariably associated with hepatomegaly induced by hypolipidaemic agents and that peroxisome proliferation is a contributory factor in increased liver weight.

Biological characteristics and functions of peroxisomes

General

In view of the striking effects of hypolipidaemic agents on the frequency, ultrastructure and enzyme activities of rat-liver peroxisomes, the last decade has witnessed increasing interest in this previously-neglected organelle. A knowledge of the biological characteristics and the physiological role of peroxisomes is of paramount importance in furthering our

understanding of the mechanism of the liver response to hypolipidaemic agents.

Occurrence and structure

Peroxisomes or microbodies, as they are often called, are cytoplasmic organelles widely present in animal and plant cells and are characterized by their content of catalase and several oxidative enzymes. Peroxisomes were described first in 1954 in mouse-kidney convoluted tubule cells and then in 1956 in rat-liver parenchymal cells (de Duve & Baudhuin, 1966; Masters & Holmes, 1977). The identification of peroxisomes was facilitated by the 3,3'-diaminobenzidine method introduced by Novikoff & Goldfischer (1969). The method relies on the cytochemical demonstration of the presence of catalase which effects peroxidatic oxidation of 3,3'-diaminobenzidine in the presence of H_2O_2, yielding a dark-brown product visible under the light microscope or, after osmication, an electron-opaque compound detectable under the electron microscope.

Rat-liver peroxisomes are usually spherical or oval in shape (approximately 0·5 μm in diameter), surrounded by a single limiting membrane containing a finely granular matrix with a characteristic electron-dense central core or crystalloid, associated with the presence of insoluble urate oxidase. However in some organs (e.g. human kidney) peroxisomes lack urate oxidase but contain some nucleoid structures; human-liver peroxisomes are devoid of both urate oxidase and nucleoids. The triple-layered membrane of peroxisomes is thinner than that of lysosomes and is comparable in thickness to that of the endoplasmic reticulum. The peroxisomal membrane is highly permeable to small molecules such as sucrose, substrates (including H_2O_2) and inorganic ions, in contrast to lysosomes which exhibit low permeability to these substrates (de Duve & Baudhuin, 1966; Masters & Holmes, 1977).

Physiological function

de Duve & Baudhuin (1966) speculated that peroxisomes (1) play a protective role in the disposal of excess H_2O_2 concentrations, which are otherwise injurious to cell components, (2) participate in specific metabolic pathways, e.g. gluconeogenesis and (3) play a role in energy production and oxidative metabolism.

Since 1966, the metabolic role of peroxisomes has become more clearly defined with progress on the determination of the enzyme content of this organelle. Thus although catalase was long regarded as the enzyme of major importance, recent studies in rats have revealed the presence of peroxisomal enzymes catalysing the β-oxidation of fatty acids, a finding having far-reaching consequences in lipid metabolism (Lazarow, 1978; Lazarow & de Duve, 1976), as will be discussed later.

Peroxisomal enzymes so far demonstrated in rat liver are listed in Table 3 and their functions will now be described briefly. Peroxisomes contain (1) oxidase enzymes that reduce oxygen to H_2O_2 with attendant oxidation of a substrate RH_2 to R, and (2) catalase which performs both catalatic and peroxidatic functions with H_2O_2 as follows:

$$\text{Catalatic: } H_2O_2 + H_2O_2 \rightarrow 2H_2O + O_2$$

$$\text{Peroxidatic: } H_2O_2 + RH_2 \rightarrow 2H_2O + R$$

It would appear that H_2O_2 generated within the peroxisome reacts preferentially with peroxisomal catalase, although the mode of removal of H_2O_2 by extraperoxisomal systems is unclear (Masters & Holmes, 1977). Catalase has been said to account for 16% (Goldfischer, Roheim, Edelstein & Essner, 1971) or 40% (de Duve & Baudhuin, 1966) of peroxisomal proteins, 50–66% of which however still await identification (Masters & Holmes, 1977).

L-α-Hydroxyacid oxidase catalyses the oxidation of L-α-hydroxyacids to produce the corresponding α-ketoacids and H_2O_2. D-Amino acid oxidase catalyses the oxidative deamination of D-amino acids and is localized in both the cytosol and peroxisomal fractions. Urate oxidase (uricase) catalyses the oxidation of urate with molecular O_2 as the only known oxygen acceptor. The conversion of urate to allantoin represents the final step in the metabolic degradation of the purine ring in many mammalian species, resulting

Table 3. *Enzymes demonstrated in rat-liver peroxisomes*

Enzyme	References*
Catalase	1, 2
L-α-Hydroxyacid oxidase	1, 2
D-Amino acid oxidase	1, 2
Urate oxidase,	1, 2
Glutamate-glyoxylate aminotransferase	1
Hydroxypyruvate/glyoxylate reductase	1
NAD^+-α-glycerophosphate dehydrogenase	1, 3
Carnitine acetyltransferase	1
Carnitine acyltransferases (short and medium-chain)	1, 4
Crotonase (enoyl-CoA hydratase)	5
β-Hydroxybutyryl-CoA dehydrogenase	5
Thiolase	5
Palmitoyl-CoA-dependent NAD^+ reducing activity	5, 6

*References: (1) Masters & Holmes (1977); (2) Leighton, Poole, Beaufay, Baudhuin, Coffey, Fowler & de Duve (1968); (3) Gee, McGroarty, Hsieh, Wied & Tolbert (1974); (4) Markwell, McGroarty, Bieber & Tolbert (1973); (5) Lazarow (1978); (6) Lazarow & de Duve (1976).

in the formation of the excretory product, allantoin. Man and certain sub-human primates are devoid of urate oxidase activity (Masters & Holmes, 1977).

Microperoxisomes

Microperoxisomes, as the name implies, are smaller organelles than peroxisomes. The microperoxisome is a roughly circular or elongated cytoplasmic body (diameter 0·15–0·25 μm), delimited by a tripartite membrane which is continuous by numerous slender channels with the SER. It has a moderately electron-opaque matrix but lacks the nucleoid characteristic of many peroxisomes. Microperoxisomes are present in many mammalian cell types including rat and human hepatocytes (Masters & Holmes, 1977; Novikoff & Novikoff, 1973; Novikoff, Novikoff, Davis & Quintana, 1973a; Novikoff, Novikoff, Quintana & Davis, 1973b). Whilst normal rat liver contains both anucleoid microperoxisomes and nucleoid-containing peroxisomes, normal human-liver hepatocytes contain only anucleoid cytoplasmic organelles (Novikoff *et al.* 1973b).

Microperoxisomes possess both catalase and some poorly-defined oxidase activity. They should not be regarded as immature forms of peroxisomes (Masters & Holmes, 1977). They may exhibit more specific spatial relationships with cellular inclusions such as stored fat droplets, lipofuscin and pancreatic zymogen granules (Masters & Holmes, 1977; Novikoff & Novikoff, 1973; Novikoff *et al.* 1973a,b).

Ribosomes are lacking where the endoplasmic reticulum is connected to microperoxisomes by slender channels, regardless of the abundance or scarcity of endoplasmic reticulum in the area (Novikoff *et al.* 1973a,b).

The metabolism, transport and storage of lipids are major functions of many cell types possessing large numbers of microperoxisomes (Novikoff & Novikoff, 1973). Molecular interchange may occur between microperoxisomes, endoplasmic reticulum and lipid droplets. Pancreatic zymogen appears to be free of lipid and, in view of the possibility that microperoxisomes and adjacent endoplasmic reticulum are related metabolically to adjacent zymogen granules, perhaps the endoplasmic reticulum and microperoxisomes are somehow involved in lipid removal from the zymogen (Novikoff & Novikoff, 1973; Novikoff *et al.* 1973a,b).

Role of peroxisomal catalase in lipid metabolism

The liver mitochondrion was regarded as the key site of fatty acid oxidation until hypolipidaemic-drug studies, which highlighted the physiological importance of liver peroxisomes, indicated otherwise and prompted a critical look at the role of peroxisomal catalase in lipid metabolism. Although various observations indicate the importance of catalase in lipid metabolism, other observations are not compatible with a simple hypothesis linking elevated catalase with a decrease in serum cholesterol/lipids.

The lines of evidence suggesting a relationship between these two events can be summarized as follows (Masters & Holmes, 1977): (1) A striking increase in peroxisome and catalase synthesis occurs during plant germination when stored lipids are converted to carbohydrate (Goldfischer *et al.* 1971; Masters & Holmes, 1977); (2) bovine hepatic catalase produces hypocholesterolaemia in man and rabbits and inhibits hepatic cholesterol synthesis (Goldfischer *et al.* 1971; Masters & Holmes, 1977; Reddy *et al.* 1974); (3) compounds structurally related to clofibrate with a similar hypolipidaemic propensity are effective in elevating liver-catalase activity and peroxisome numbers; (4) peroxisomes and microperoxisomes are frequently encountered in cells involved in the active metabolism of lipids, such as brown adipose tissue and adrenocortical cells; (5) a mutant acatalasemic mouse strain (Cs^b), a strain with an unstable catalase gene, shows reduced basal serum triglyceride, possibly as a result of the degraded catalase acting as an active peroxidase (Goldfischer *et al.* 1971), although this possibility has been refuted in view of an abnormality of hepatic lipogenesis in this mutant strain (Masters & Holmes, 1977; Reddy *et al.* 1974); (6) *N*-allyl-*N*-isopropylacetamide, an agent that blocks catalase synthesis, has been shown to produce marked hyperlipidaemia in rats (Goldfischer *et al.* 1971; Masters & Holmes, 1977); (7) aminotriazole administration, which inactivates catalase, is associated with an increase in serum cholesterol (Hruban, Mochizuki, Gotoh, Slesers & Chou, 1974b).

In contrast, other findings, which are summarized below, show a lack of association between hepatic catalase activity and serum cholesterol/triglyceride levels (Masters & Holmes, 1977): (1) Clofibrate treatment of thyroidectomized rats increases catalase activity and peroxisome numbers, despite the abolition of the hypocholesterolaemic action of clofibrate; (2) in one study, female mice fed clofibrate showed no hypocholesterolaemic response even after 5 wk but serum triglyceride was reduced; (3) when clofibrate-treated mice were also given aminotriazole, catalase was reduced to abnormally low levels but serum triglyceride was altered little from its depressed state; (4) *N*-allyl-*N*-isopropylacetamide given to normal mice increased serum triglycerides in the final stages of treatment but no correlation was found between liver catalase and serum triglyceride levels in the intervening period; (5) when liver catalase was reduced by a combination of aminotriazole and *N*-allyl-*N*-isopropylacetamide, there was a paradoxical effect on serum triglycerides—in normal mice, triglyceride fell to a level similar to that of clofibrate controls, whereas in clofibrate-treated mice the levels rose almost to the untreated normal control level; (6) hypocholesterolaemia in rats induced by boxidine and haloperidol occurs in the absence of an increase in catalase or peroxisomes (Hruban *et al.* 1974b).

Thus there is no clear association between changes in catalase activity and certain changes in lipid metabolism although, as we shall discuss later, there exists a close relationship between peroxisome proliferation and hypolipidaemic action in rodents.

The increase in peroxisomal numbers in the livers of clofibrate-treated animals is not dependent upon or necessarily proportional to the catalase content (Masters & Holmes, 1977; Reddy, Chiga, Bunyaratvej & Svoboda, 1970). It is noteworthy that after clofibrate treatment, the catalase increase in mouse liver is accounted for by decreased degradation rather that by increased synthesis (Masters & Holmes, 1977), the reverse situation however being found in rat liver (Reddy, Chiga & Svoboda, 1971). It is possible that

aged catalase in the cytosol may act to trigger increased synthesis of peroxisomal catalase (Masters & Holmes, 1977).

Since peroxisomal catalase cannot account for the hypolipidaemic action of various drugs, other theories have been proposed to explain the mechanism of action, e.g. thyroxine response and peroxisomal oxiddases, the merits of which are discussed later.

Peroxisomal abnormalities in human diseases

Peroxisomal abnormalities have been observed in the livers of humans with certain diseases such as Zellweger's cerebrohepatorenal syndrome (Goldfischer, Johnson, Essner, Moore & Ritch, 1973) or Wilson's disease (Sternlieb & Quintana, 1977) but the significance of the peroxisomal abnormalities in relation to the disease processes is unknown.

Effect of hypolipidaemic drugs/agents on liver peroxisomes in rodents

Increase in peroxisomal numbers (peroxisomal proliferation)

Clofibrate was the first hypolipidaemic drug found to induce a striking increase in the number of liver peroxisomes in rats (Hess *et al.* 1965; Svoboda & Azarnoff, 1966; Svoboda *et al.* 1967). Peroxisomal proliferation is commonly associated with other hypotriglyceridaemic drugs/agents but not with hypocholesterolaemic compounds (Table 2). Peroxisome proliferation is sustained as long as the hypolipidaemic drug is administered (Hess *et al.* 1965; Reddy, Bunyaratvej & Svoboda, 1969; Svoboda & Azarnoff, 1966)—even up to 25 months of treatment (Reddy & Rao, 1977a).

An increase in the number of hepatic peroxisomes induced by clofibrate is first apparent 24 hr after administration of an oral dose of 500 mg/kg to male rats (Hess *et al.* 1965) or 3 days after the feeding of a dietary level of 0·25% to male F-344 rats (Svoboda *et al.* 1967). The increase was sustained in rats during 14 daily oral doses of 500 mg/kg (Hess *et al.* 1965) or for 30 days on a 0·25% clofibrate diet, but maintenance of treatment on 0·25% after 30 days did not elicit any further increase in the number of peroxisomes, although by wk 6 atypical forms were more prevalent than before (Svoboda *et al.* 1967). The rapid increase in peroxisome numbers contrasts with the relatively slow return to normality following cessation of clofibrate treatment in rats (Hess *et al.* 1965; Svoboda *et al.* 1967), 3 wk being required to attain normal peroxisomal numbers after 4 or 8 wk on a 0·25% clofibrate diet (Svoboda *et al.* 1967).

In the normal rat hepatocyte, the ratio of peroxisomes to mitochondria is approximately 1:8, but after clofibrate treatment the number of peroxisomes per cell increases tenfold although the number of peroxisome-containing nucleoids is not proportionally increased (Svoboda & Azarnoff, 1966).

Changes in peroxisome structure

Peroxisomes undergo various structural changes in response to hypolipidaemic agents. These include loss of a nucleoid, changes in matrix density and the appearance of matrix tubules or striations, marginal plates and elongated profiles.

After 14 daily oral doses of 500 mg clofibrate/kg to male rats, liver peroxisomes were frequently without cores but no alteration was seen in the single membrane or appearance of the finely granular matrix (Hess *et al.* 1965). The accumulating peroxisomes, some in clusters, were distributed throughout the cytoplasm and there was no obvious association with other cytoplasmic components, except that the peroxisomal membrane was in part connected to the SER by means of short hook-like or finger-like extensions.

More detailed study in male CFE or F-344 rats given clofibrate at 0·25–0·5% in the diet for 3–30 days (Svoboda & Azarnoff, 1966) or in male F-344 rats given clofibrate at 0·25% for between 3 days and 10 wk (Svoboda *et al.* 1967) confirmed the presence of a large proportion of peroxisomes, abnormal in size and shape; these were not seen at 12 hr but were apparent 3 days after treatment commenced. Whilst peroxisomes of normal size with typical crystalloid nucleoids were present in significant numbers, many peroxisomes were abnormal in size and shape, lacked nucleoids, had a flocculent, less dense matrix and were attached to membranes of the SER. When nucleoids were present they were usually small and eccentric and lacked normal crystalloid structure. By day 30 peroxisomes frequently assumed abnormal shapes with elongated extensions, several linear striations were prominent in the matrix and the continuity of peroxisomes with vesicles of SER became increasingly conspicuous. By wk 8, ill-defined diffuse densities lacking a distinct limiting membrane were present.

Typical ultrastructural changes were seen in the liver peroxisomes of male F-344 rats given the hypolipidaemic agent RMI 14,514 (Svoboda, 1978). No alterations in peroxisome structure were apparent in male F-344 rats fed 0·1% clofibrate in the diet for 3–10 wk (Svoboda *et al.* 1967).

Interesting differences in the pattern of ultrastructural changes in rat-liver peroxisomes were seen following administration of various compounds. Thus acetylsalicylic acid and related compounds produced accumulation of fibrillar material in the matrix, terephthalanilides produced enlargement of the crystalloid, whilst hexahydrosalicylic acid elicited extrusion of the crystalloid from the peroxisome (Hruban, Swift & Slesers, 1966). Tetracycline, azaserine and the hepatocarcinogens thioacetamide and *N,N'*-2,7-fluorenylenebisacetamide also produced abnormal inclusions in peroxisomes (Svoboda & Azarnoff, 1966).

Species differences in peroxisome proliferation

Clofibrate-induced peroxisome proliferation has been demonstrated in the rat, mouse, dog and hamster but not in limited studies in the guinea-pig, chicken, rabbit or monkey (Svoboda *et al.* 1967); in these last four species, SER proliferation does occur, however, albeit to varying extents (see also Table 4).

No evidence of peroxisome proliferation has been obtained in humans treated with fenofibrate (Blane & Pinaroli, 1980) or with clofibrate (Hanefeld *et al.* 1980) or in monkeys treated orally for 1 yr either with fenofibrate or clofibrate (Blane & Pinaroli, 1980).

Table 4. *Species differences in hepatic peroxisome proliferation induced by various hypolipidaemic drugs*

Compound	Species	Strain	Sex	Treatment	Peroxisome proliferation	References*
Clofibrate	Rat	?	M	500 mg/kg/day orally for 14 days	+	1
		CFE	M	0·25–0·5% in diet for 3–30 days	+	2
		F-344	M	0·25–0·5% in diet for 3–30 days	+	2
			M	0·25% in diet for 3 days–21 wk	+	3, 4
		Sprague–Dawley	M/F	250 mg/kg bw/day in diet for 91 days	+	5
		F-344	M	0·1% in diet for 3 or 10 wk	–	3
			F	0·25% in diet for 2 or 4 wk	–	3, 4
	Mouse	C3H	M	0·25–0·5% in diet for 3–30 days	+	2
			M	0·25% in diet for 9 days–5 months	+	3
		Swiss-Webster	M	0·25–0·5% in diet for 3–30 days	+	2
		Acatalasemic Cs[b]	M	NK	+	6
			F	NK	+	6
		Wild-type Cs[a]	M	NK	+	6
			F	NK	–	6
	Dog		M	25 mg/kg/day for 26 days	+	3
	Hamster		M	0·25% in diet for 2 wk	+	3
	Guinea-pig		M	0·25% in diet for 2 wk	–	3
	Chicken		M	0·25% in diet for 3 wk	–	3
	Rabbit		M	0·25% in diet for 2 wk	–	3
	Squirrel Monkey		M	75 mg/kg/day orally for 22 days	–	3
	Monkey	Rhesus	M/F	200 mg/kg/day orally for 1 yr	–	7
	Man		?	NK	–	8
Fenofibrate	Rat	Sprague–Dawley	M	100 mg/kg bw/day in diet for 28 days	+	5
		Wistar	M	30–300 mg/kg/day orally for 1 wk	+	9
			M	3 or 10 mg/kg/day orally for 1 wk	–	9
	Monkey	Rhesus	M/F	12–200 mg/kg/day orally for 1 yr	–	7
	Man		?	2 months–7 yr	–	7
Nafenopin	Rat	F-344	M	0·0125–0·25% in diet for 1–8 wk	+	10
			F	0·0125–0·25% in diet for 1–8 wk	+	10
	Mouse	Wild-type Cs[a]	M	0·125 or 0·25% in diet for 1–8 wk	+	10
			F	0·125 or 0·25% in diet for 1–8 wk	+	10
		Acatalasemic Cs[b]	M	0·125 or 0·25% in diet for 1–8 wk	+	10
			F	0·125 or 0.25% in diet for 1–8 wk	+	10
Probucol	Rat	Sprague–Dawley	M/F	500 mg/kg bw/day in diet for 91 days	–	5
	Monkey	Rhesus	M/F	Up to 500 mg/kg/day for 8 yr	–	11

+ = Effect – = No effect NK = Not known

*References: (1) Hess *et al.* (1965); (2) Svoboda & Azarnoff (1966); (3) Svoboda *et al.* (1967); (4) Svoboda *et al.* (1969); (5) Barnard *et al.* (1980); (6) Reddy *et al.* (1969); (7) Blane & Pinaroli (1980); (8) Hanefeld *et al.* (1980); (9) G. F. Blane (unpublished report 1979); (10) Reddy *et al.* (1974); (11) Molello *et al.* (1980).

Sex differences in peroxisome response

The ability of male but not female F-344 rats fed 0·25% clofibrate for up to 4 wk to exhibit increased numbers of liver peroxisomes and structural alterations in these organelles was initially attributed to differences in liver-catalase levels, which are greater in males than females (Svoboda *et al.* 1967). With clofibrate, the lack of response in females in respect of both peroxisome proliferation and liver catalase applied not only to F-344 rats but also to C3H mice and wild-type (Cs^a) mice (Reddy *et al.* 1969; Svoboda, Azarnoff & Reddy, 1969; Svoboda *et al.* 1967). Subsequently, the dependence of peroxisome proliferation on the male sex hormone in adult rats was indicated by the absence of a peroxisome response in castrated males given clofibrate plus oestradiol benzoate and the presence of a peroxisome response in ovariectomized females given clofibrate plus testosterone propionate (Svoboda *et al.* 1969).

The sex difference in peroxisome response seen with clofibrate in adult rats was not manifest, however, with nafenopin. As can be seen from Table 4, both sexes of the F-344 rat, wild-type (Cs^a) mouse and acatalasemic (Cs^b) mouse showed peroxisome proliferation, and this was accompanied in the F-344 rat and Cs^a mouse by elevated hepatic catalase activity (Reddy *et al.* 1974). Also, no sex difference was seen with the hypolipidaemic agent, RMI 14,514, in respect of the increased peroxisomal enzyme levels in F-344 rats (Svoboda, 1978). No sex difference in peroxisome proliferation was apparent in foetal or neonatal rats of mothers treated with clofibrate (Svoboda *et al.* 1969).

Dose-response relationship in peroxisome proliferation

In male F-344 rats, peroxisome proliferation was not seen when 0·1% clofibrate was given for 3 or 10 wk but was apparent at the 0·25% dietary level (Svoboda *et al.* 1967).

With nafenopin, the increase in peroxisome numbers in F-344 rats was relatively low at 0·0125% in the diet, compared with levels of 0·125 and 0·25% administered for 4 wk. Peroxisomal proliferation at the 0.125% level was as marked as that seen at 0·25% in F-344 rats and wild-type Cs^a and acatalasemic Cs^b mice. However, ultrastructural changes in the peroxisomes of F-344 rats were seen at the lowest level of nafenopin (0·0125%) and were similar to those seen with clofibrate (Reddy *et al.* 1974).

With fenofibrate, peroxisome proliferation was not apparent at 13 mg/kg/day but was demonstrable at 60 and 200 mg/kg/day over 28 days in rats (S. C. Price *et al.* unpublished observations 1981). In another fenofibrate study, peroxisome proliferation was evident at 60 mg/kg body weight/day but not at 10 mg/kg/day following administration in the diet of rats for 117 wk (Blane & Pinaroli, 1980).

Hormonal influence on peroxisome response and hypolipidaemic action

In male rats neither thyroidectomy nor adrenalectomy had any significant effect on clofibrate-induced peroxisome proliferation and elevated liver catalase activity but both procedures can abolish clofibrate-induced hypolipidaemia. Whilst neither thyroid nor adrenal hormones appear therefore to be necessary for clofibrate-induced peroxisome proliferation, both hormones were considered to play a possible role in the hypolipidaemic effect of clofibrate (Svoboda *et al.* 1969).

Reversal of peroxisome response following drug withdrawal

During the 7-day period after cessation of a 6-wk treatment with a 0·25% nafenopin diet, rat-liver peroxisome numbers gradually declined, reaching normality 14 days after drug withdrawal (Reddy *et al.* 1974). In the first week after withdrawal, the peroxisomes underwent significant changes, characterized mainly by dissolution of the microbody matrix and the appearance of nucleoid cores in dilated endoplasmic-reticulum membranes or occasionally in the hyaloplasm. There was no evidence of peroxisome incorporation into lysosomes or into autophagic vacuoles. It would appear that nafenopin withdrawal results in a cessation of the enhanced synthesis of peroxisomal proteins and the loss of peroxisome numbers results from the progressive reduction in the peroxisomal matrix proteins in endoplasmic reticulum channels.

With clofibrate, increased peroxisome numbers still persisted 2 wk after cessation of treatment (500 mg/kg/day orally for 14 days) in rats, despite the return to normal liver weight; in contrast to the slow decline in elevated peroxisome numbers, proliferation of the SER regressed rapidly after treatment was withdrawn (Hess *et al.* 1965). These results were not fully confirmed in a subsequent study (Svoboda *et al.* 1967), in which rats were returned to the basal diet after 4 or 8 wk on a 0·25% clofibrate diet. Whilst a gradual decline to normality was again seen in the peroxisome population over 3 post-treatment weeks, proliferation of the SER had not completely regressed by the end of this period.

Relationship between peroxisome proliferation and hypolipidaemic action in rodents

Earlier we discussed the role of peroxisomal catalase in lipid metabolism and concluded that there is no definitive association between catalase levels and the hypolipidaemic response.

The striking ability of clofibrate and other hypolipidaemic aryloxyisobutyrate derivatives to induce hepatic peroxisome proliferation in rats and mice led to the suggestion of an association between hypolipidaemic action and peroxisome-proliferation induction (Reddy & Krishnakantha, 1975; Reddy, Svoboda & Azarnoff, 1973). This viewpoint has gained further support from the findings that hypolipidaemic agents structurally-unrelated to clofibrate, such as Wy-14,643 (Reddy & Krishnakantha, 1975), tibric acid (Reddy & Krishnakantha, 1975), the plasticizer di-(2-ethylhexyl) phthalate (Lake *et al.* 1975; Moody & Reddy, 1978b; Reddy, Moody, Azarnoff & Rao, 1976b) and acetylsalicylic acid (Hruban *et al.* 1966; Ishii & Suga, 1979) also act as peroxisome proliferators in rats or mice. The fact that analogues of Wy-14,643 (Reddy *et al.* 1979) and certain compounds related to di-(2-ethylhexyl) phthalate (Moody & Reddy, 1978b) are devoid of hypolipidaemic activity and lack the ability to induce peroxisome proliferation is also compatible

with this hypothesis. So is the finding that in nafenopin-treated rats serum triglyceride levels return to normal within 7–10 days after drug withdrawal and this is paralleled by reductions in catalase activity and peroxisome numbers (Reddy *et al.* 1974).

However it is clear that peroxisome proliferation and hypolipidaemic action are closely but not invariably inter-related. Thus of the various hypolipidaemic (triglyceride-lowering) agents examined (Table 2), only one compound, 3,9-di-3-pyridyl-2,4,8,10-tetraoxaspiro-5,5-undecane, failed to induce hepatic peroxisome proliferation in rats although it succeeded in causing SER proliferation. Moreover studies on clofibrate have shown that thyroidectomy can abolish the hypolipidaemic effect without affecting peroxisome proliferation in rats and that gonadectomy has little effect on hypolipidaemic action although peroxisome proliferation under certain experimental conditions is dependent upon the male sex hormone (Svoboda *et al.* 1969). In another study, it has been claimed that thyroidectomy does not abolish the hypolipidaemic effect in clofibrate-treated animals and therefore this argument cannot be used to dissociate the two responses (Reddy & Krishnakantha, 1975).

In discussing correlations between hypolipidaemic action and peroxisome proliferation, it is important to draw a distinction between compounds lowering both serum triglycerides and serum cholesterol (e.g. clofibrate) and those lowering serum cholesterol alone (e.g. probucol). Hypocholesterolaemic drugs like the much-studied probucol and other less-studied hypocholesterolaemic agents like benzmalacene, benzyl-*N*-benzylcarbethoxyhydroxamate, dextran, bovine catalase and 2,4-dichlorophenoxyacetic acid exert no effect on the liver peroxisome population (Table 2). In contrast, other hypocholesterolaemic agents such as chlorcyclizine, *N*-(benzyloxy)-*N*-(3-phenylpropyl)-acetamide and 1-benzylimidazole have been found to induce peroxisomal proliferation in rat liver (Table 2), but the significance of this finding is difficult to interpret since liver enlargement in this experiment was accompanied by significant growth retardation and complicating histological changes in the liver, uncharacteristic of hypolipidaemic peroxisome proliferators (Hruban *et al.* 1974b).

Response of peroxisomal enzymes to hypolipidaemic agents

Various hypolipidaemic drugs/agents that are known to induce liver enlargement and peroxisome proliferation are also capable of influencing peroxisomal enzyme activities. In addition to the marker enzyme, catalase, peroxisomes contain several H_2O_2-generating oxidases as well as carnitine acetyltransferase and various enzymes involved in fatty-acid oxidation (Table 3). These enzymes, whether assayed in whole-liver homogenate or in peroxisomal fractions, show a varying response to hypolipidaemic drugs, as can be seen from Table 5. It is clear that neither catalase nor the group of oxidases (urate oxidase, D-amino acid oxidase, L-α-hydroxyacid oxidase) is influenced as markedly as carnitine acetyltransferase or the enzymes involved in the β-oxidation of fatty acids. The latter group and carnitine acetyltransferase exhibit striking increases in activity (Table 5).

Lazarow & de Duve (1976) demonstrated that normal rat-liver peroxisomes oxidize palmitoyl-CoA with the reduction of O_2 to H_2O_2 and NAD to NADH. In clofibrate-treated rats, palmitoyl CoA-dependent NAD-reducing activity was increased nearly sevenfold compared with a 53% increase for catalase. Peroxisomal protein in clofibrate-treated rats was five times greater than that of control rats. Soon afterwards, Lazarow (1978) showed that peroxisomes carry the full complement of enzymes concerned in the β-oxidation of long-chain fatty acids. Thus peroxisomes (as well as mitochondria) contain low levels of crotonase, β-hydroxybutyryl-CoA dehydrogenase and thiolase and these levels are considerably elevated in clofibrate-treated rats. Moreover peroxisomes oxidize palmitoyl-CoA to acetyl-CoA which accumulates as the end-product of hepatic peroxisomal β-oxidation. The fate of peroxisomal acetyl-CoA is unknown but the presence of carnitine acetyltransferase in peroxisomes is suggestive of a role in transporting 'active acetate' to the mitochondria for further oxidation. Although both peroxisomes and mitochondria catalyse β-oxidation it would appear that in clofibrate-treated rats most of the hepatic palmitoyl-CoA oxidation occurs in peroxisomes (Lazarow, 1978). The induction of the enzymes of the β-oxidation system for fatty acyl-CoA was also demonstrated in the liver peroxisomes of rats treated with di-(2-ethylhexyl) phthalate (Osumi & Hashimoto, 1979).

In summary, therefore, there appear to be three types of response of peroxisomal enzymes to clofibrate (Goldenberg, Hüttinger, Kampfer, Kramar & Pavelka, 1976) and other hypolipidaemic drugs (Table 5):

(1) With oxidases (like urate oxidase), synthesis is not increased by the proliferative stimulus of the hypolipidaemic drug and thus these enzymes exhibit a lower specific activity in the peroxisomal fraction. (The behaviour of urate oxidase in particular is consistent with the numerical increase of coreless peroxisomes in response to hypolipidaemic drugs.)

(2) With some enzymes (like catalase), synthesis is increased in proportion to the formation of total peroxisomal protein so that the specific activity of the enzyme remains constant although the rise in activity per gram of liver reflects only the increase in the number of peroxisomes.

(3) With enzymes such as carnitine acetyltransferase and especially those involved in the β-oxidation of fatty acids, synthesis is markedly and selectively induced by the hypolipidaemic agent.

Mutagenicity and carcinogenicity studies on hypolipidaemic drugs/agents

Mutagenicity studies

Fenofibric acid (the major metabolite of fenofibrate) and to a lesser extent fenofibrate were tested in various mutagenicity assay systems (Blane & Pinaroli, 1980). Neither compound elicited any significant adverse effects in *in vitro* microbial tests, *in vitro* mouse-lymphoma cell assays, sister-chromatid

Table 5. *Effect of hypolipidaemic drugs/agents on rodent-liver peroxisomal enzymes*

Compound	Treatment (% in diet except where marked†)	Species, strain, sex	Hepatic fraction assayed	Enzyme activity (% of controls)							References*
				Urate oxidase	D-Amino acid oxidase	L-α-Hydroxy acid oxidase	Catalase	Palmitoyl CoA NAD^+, red. act.	Carnitine acetyl-transferase	NAD^+-α-GPD	
Clofibrate	0·25%; 3 wk	Rat SD (M)	Hom				264				1
	0·25%; 3 wk	Rat SD (F)	Hom				133				1
		Rat (M)	Perox		20	20					1
	20†; 1 or 2 wk	Rat (M)	Perox				144	202–225			2
	0·75%; 2–5 wk	Rat SD (M)	Hom	78–101			200–267		1155–1604		3
			Perox	33–42			88–102		476–577		3
			Mit						616–698		3
	0·5%; 6 days	Rat F-344 (M)	Hom				150	900			4
	0·25%; 2–14 days	Rat F-344 (M)	Hom				141–210				5
	300†; 1 wk	Rat (M)	Hom	42			127			613	7
	0·4%; 2 wk	Rat H (M)	Hom				151	1568			16
			Mit							375	16
	0·125%; 4 wk	Mouse SW (M)	Hom	173			98		350		6
	0·25%; 4 wk	Mouse SW (M)	Hom				191		690	140	6
Fenofibrate	0·05%; 2 wk	Rat H (M)	Hom				134	2247			16
			Mit							377	16
Mg-CPIB	20†; 1 or 2 wk	Rat (M)	Perox				121	253–393			2
Nafenopin	0·125, 0·25%; 4 wk	Rat F-344 (M + F)	Hom				197–265				8
	0·125%; 4 wk	Mouse SW (M)	Hom	147			168		2642	243	6
Wy-14,643	0·125, 0·25%; 2 wk	Rat F-344 (M)	Hom				213, 205				9
	0·1%; 6 days	Rat F-344 (M)	Hom				145	860			4
	0·125, 0·25%; 2 wk	Mouse SW (M)	Hom				237, 233				9
	0·125%; 4 wk	Mouse SW (M)	Hom	43			160		1760	267	6
Tibric acid	0·125, 0·25% 2 wk	Rat F-344 (M)	Hom				205, 221				9
	0·1%; 6 days	Rat F-344 (M)	Hom				155	650			4
	0·05%; 2 wk	Rat H (M)	Hom				136	392			16
			Mit							782	16
	0·125, 0·25%; 2 wk	Mouse SW (M)	Hom				239–269				9
	0·125%; 4 wk	Mouse SW (M)	Hom	56			215		1462	240	6
Acetylsalicylic acid	1%; 2 wk	Rat W (M)	Hom	121			117	378	924		10
			Mit	105			116	397			10
			Sup	105			147	104			10

Di-(2-ethylhexyl) phthalate	0·5–4·0%; 1–4 wk	Rat F-344 (M)	Hom				142–217		2733‡	11
	2%; 3 wk	Rat F-344 (M)	Hom				200		1696	12
	2%; 8 wk	Rat Wistar (M)	Hom	123			313	2305		13
	2, 4%; 1–4 wk	Mouse SW (M)	Hom				181–184		1035‡	11
Di-(2-ethylhexyl) adipate	2%; 3 wk	Rat F-344 (M)	Hom				195		1137	12
Di-(2-ethylhexyl) sebacate	2%; 3 wk	Rat F-344 (M)	Hom				179		1322	12
RMI 14,514	150†; 1 wk	Rat F-344 (M)	Hom	184	217	175	209			14
		(F)	Hom	167	187	150	192			14
Haloperidol§	0·18%; 6 wk	Rat SD (M)	Hom	170	123		98			15
W-1372§	0·25%; 6 wk	Rat SD (M)	Hom	389	316		151			15
1-Benzyl-imidazole§	0·18%; 6 wk	Rat SD (M)	Hom	214	204		237			15
Boxidine§	0·008%; 6 wk	Rat SD (M)	Hom	94	115		119			15

(M) = Male (F) = Female SD = Sprague–Dawley SW = Swiss–Webster W = Wistar H = Hooded GPD = Glycerophosphate dehydrogenase
Hom = Homogenate Perox = Peroxisomal Mit = Mitochondrial Sup = Supernatant

*References: (1) Svoboda *et al.* (1969); (2) Paltauf & Magnet (1979); (3) Goldenberg *et al.* (1976); (4) Lazarow (1977); (5) Reddy *et al.* (1971); (6) Moody & Reddy (1978a); (7) Hess *et al.* (1965); (8) Reddy *et al.* (1974); (9) Reddy & Krishnakantha (1975); (10) Ishii & Suga (1979); (11) Reddy *et al.* (1976b); (12) Moody & Reddy (1978b); (13) Osumi & Hashimoto (1979); (14) Svoboda (1978); (15) Hruban *et al.* (1974b); (16) Holloway & Orton (1980).

†mg/kg/day, orally.

‡At 4% in diet only.

§Cholesterol-lowering agents; the other hypolipidaemic compounds are trigylceride-lowering agents.

exchange tests, *in vitro* BALB/3T3 cell-transformation assays and tests involving DNA repair in HeLa cells, covalent binding with rat-liver DNA and proliferation of human liver cells in culture.

Negative results were also obtained with clofibrate, nafenopin, SaH-42-348, tibric acid, Wy-14,643 and BR-931 in the Salmonella/microsome assay or in the lymphocyte [^{3}H]thymidine incorporation into replicating DNA assay. Thus it would appear that hypolipidaemic drugs do not interact with and damage cellular DNA (Warren *et al.* 1980). These workers, however, raise the question as to whether mutagenic metabolites are generated by hypolipidaemic drug-induced rodent-liver peroxisomes. Support for this possibility has come from the selective induction of H_2O_2-generating enzymes in rodents (Table 5). The negative findings obtained in the mutagenicity assays on hypolipidaemic drugs are not inconsistent with this possibility, since they are not designed to detect mutagenic reactive species that are formed endogenously in the rodent liver.

von Däniken *et al.* (1981) also furnished evidence for the lack of genotoxic activity of fenofibrate. Liver nuclear DNA and protein were examined 10 hr after administration of ^{14}C-labelled fenofibric acid to rats in an oral dose of 46–206 mg/kg. Protein-binding activity was demonstrable but binding of ^{14}C to DNA was not detected.

Hepatocarcinogenicity studies of hypolipidaemic drugs/agents

Because various structurally-unrelated hypolipidaemic peroxisome proliferators induce hepatic carcinomas in mice and rats it has been suggested that hypolipidaemic compounds capable of producing sustained hepatomegaly and peroxisome proliferation will also be capable of producing liver tumours (Reddy *et al.* 1980; Reddy & Qureshi, 1979).

Although not designed as full-scale carcinogenicity studies, experiments conducted in rats and mice have provided adequate evidence that various hypolipidaemic agents, including clofibrate, nafenopin, Wy-14,643, BR-931 and tibric acid, can produce liver carcinomas in these species, the male F-344 rat being the most frequent animal model used (Table 6). Although limited group sizes and numbers have been used for each compound, a high proportion of treated animals developed hepatocellular carcinomas, some of which metastasized to the lungs.

In the case of Wy-14,643, all mice and rats surviving 14·5–16 months developed hepatocellular carcinomas, and pulmonary metastasis was seen in about one third of the animals (Reddy *et al.* 1979). Pulmonary metastasis was also seen in some liver-tumour bearing rats given clofibrate (Reddy & Qureshi, 1979) or nafenopin (Reddy & Rao, 1977a) and in some liver-tumour bearing mice given nafenopin (Reddy *et al.* 1976a).

After 18 months on a 0·1% nafenopin diet, male F-344 rats showed several small nodules in the liver; by 20–25 months hepatocellular carcinomas had developed in most animals (Reddy & Rao, 1977a). Similar results were obtained in acatalasemic Cs^b mice (Reddy *et al.* 1976a).

However, negative findings have been obtained in carcinogenicity studies on fenofibrate in mice and rats and on clofibrate in mice. In the case of fenofibrate, no hepatocellular carcinomas developed following dietary administration providing daily intakes of 50 mg/kg to Swiss mice for 96 wk or of 10 or 60 mg/kg to Sprague–Dawley rats for 117 wk (Blane & Pinaroli, 1980). In mice, liver enlargement was

Table 6. *Incidence of liver carcinomas in mice and rats fed various hypolipidaemic drugs/agents—published studies*

Compound	Species	Dietary level (%) and duration	Sex	No. of animals		Animals with tumours		References‡
				Initial	Effective*	No.	(%)†	
Clofibrate	F-344 rat	0·5%, 28 months	M	15	11	10	91	1
		0·5%, 72–97 wk and examined 72–129 wk	M	25	25?	4	16?	2
		0·25%, 26 months	M	5	5?	2§	40?	3
Wy-14,643	Cs^b mouse	0·1%, 6 months then 0·05%, 8·5 months	M	20	18	18	100	4
	F-344 rat	0·1%, 16 months	M	15	15	15	100	4
Nafenopin	F-344 rat	0·1%, 25 months	M	15	15?	11	73?	3, 5
	Cs^b mouse	0·1%, 12 months; then 0·05%, 8 months	M	20	9	9	100	7
			F	20	12	12	100	7
BR-931	Cs^b mouse	0·2%, 19 months	F	15	12	11	92	6
	F-344 rat	0·2%, 16 months	M	20	20	20	100	6
		0·05%, 19 months	M	10	10	7	70	6
Tibric acid	F-344 rat	0·2%, 7·5 months; then 0·1%, 4 months, and 0·05%, 5 months	M	35	31	30	97	6

*No. alive at onset of first liver tumour.
†Percentage in relation to effective number. No liver carcinomas were found in the control groups in any of these studies.
‡References: (1) Reddy & Qureshi (1979); (2) Svoboda & Azarnoff (1979); ((3) Reddy & Rao (1977a); (4) Reddy *et al.* (1979); (5) Reddy & Rao (1977b); (6) Reddy *et al.* (1980); (7) Reddy *et al.* (1976a).
§Well-differentiated hepatomas.
Consecutive treatments.

unaccompanied by histological change. In rats, liver enlargement and peroxisome proliferation were observed at 60 mg/kg but not at 10 mg/kg, and this study failed, at the doses used, to reveal evidence of an increased incidence of foci of altered hepatocytes or of neoplastic nodules. Whether higher doses of fenofibrate would elicit additional effects cannot be answered from these studies. The highest dose tested (60 mg/kg) is 12 times the human therapeutic dose and was selected on the basis that it was pharmacologically equivalent to a dose of 400 mg clofibrate/kg, administration of which in the diet of rats for 117 wk resulted in a high incidence of hepatocellular carcinomas—80% in males and 54% in females (Blane & Pinaroli, 1980).

With clofibrate, no hepatocellular carcinomas were found with the highest dietary intake used in two unpublished mouse studies (i.e. 350 mg/kg/day), an intake comparable with the dietary level of 0·5% which proved to be carcinogenic in rats (Table 6). In the first of these two mouse studies (Ayerst Laboratories, USA; unpublished study cited by *Japan Medical Gazette* of 20 January 1981), groups of 50 male and 50 female CD-1 mice received clofibrate in the diet to provide daily intakes of 0, 150, 250 or 350 mg/kg for 78 wk. A dose-related increase in liver weight was not accompanied by histological proliferative changes except for hyperplastic nodules in females on 350 mg/kg/day. No difference was found in either the time of onset or incidence of tumours between the control and test groups. In the second study (Imperial Chemical Industries Ltd; unpublished study cited by *Japan Medical Gazette* of 20 January 1981), groups of 51 male and 51 female C57BL/10J mice given clofibrate in daily dietary intakes of 150, 250 or 350 mg/kg for 18 months exhibited liver enlargement (dose-related) and hepatic-cell hypertrophy but no evidence of an increased incidence of tumours in the liver or at other sites, when compared with that of an untreated control group consisting of 153 males and 153 females.

In addition to hypolipidaemic drugs, certain hypolipidaemic plasticizers have been found recently to induce liver tumours in lifespan studies in mice and rats, albeit at levels 2000-fold greater than the estimated daily human intake, as follows: di-(2-ethylhexyl) phthalate in F-344 rats (both sexes) at 1·2% in diet and in B6C3F1 mice (both sexes) at the 0·3 and 0·6% dietary levels (National Cancer Institute, 1981) and di-(2-ethylhexyl) adipate in both sexes of B6C3F1 mice at the 1·2 and 2·5% dietary levels but not in F-344 rats at levels up to 2·5% in the diet (National Cancer Institute, 1980).

Wy-14,643 or clofibrate at 0·5% in the diet for 48 wk enhanced the incidence of liver tumours induced in male F-344 rats pretreated with *N*-nitrosodiethylamine for 1 wk (Reddy & Rao, 1978).

Carcinogenicity studies on probucol yielded negative results in rats and monkeys. Doses of up to 800 mg/kg/day given to rats for 2 yr and up to 500 mg/kg/day given for 8 yr to monkeys produced no effect on liver weight nor any histological or ultrastructural changes in the liver (Lepetit Pharmaceuticals Ltd., 1980; Molello, Barnard & LeBeau, 1980).

Discussion

Basis for absence of hepatotoxicity of hypocholesterolaemic drugs

Whilst hypotriglyceridaemic drugs/agents like fenofibrate, clofibrate and tibric acid induce liver damage in rodents, certain hypocholesterolaemic drugs, such as probucol, do not, and the only hepatic effect seen with probucol is a questionable proliferation of the SER.

The striking difference in toxic action between these two classes of lipid-lowering agents can be related to differences in their mechanism of pharmacological action. Probucol exerts its hypocholesterolaemic action in man by means of enhanced faecal excretion of bile acids and of a reduction of cholesterol synthesis coupled with a slight decrease in the absorption of dietary cholesterol (Miettinen, 1972). The probucol-induced reduction of serum cholesterol in hypercholesterolaemic monkeys has also been attributed to the increased excretion of faecal bile acids (Kritchevsky, 1980a). Moreover, evidence has been obtained from liver biopsy studies in man (Miettinen, 1972) and from tissue distribution studies in animals (Kritchevsky, 1980a) that the hypocholesterolaemic effect of probucol is not caused by a redistribution of cholesterol to other tissues. Probucol has no effect on serum triglycerides and the hypocholesterolaemic effect in both man and rats is due to a reduction mainly in LDL-cholesterol, i.e. that in the low-density-lipoprotein fraction, and to a small extent in HDL-cholesterol, present in the high-density lipoprotein fraction (Lepetit Pharmaceuticals Ltd, 1980).

Studies on clofibrate and fenofibrate have shown that in hypercholesterolaemic rats the rate of cholesterol elimination is a determining factor in serum cholesterol reduction whilst in normocholesterolaemic rats inhibition of cholesterol biosynthesis is the determining factor (G. F. Blane, unpublished report 1979).

Thus the reduction in serum cholesterol encountered with probucol does not impose metabolic demands on the rodent liver as are encountered with the hypotriglyceridaemic agents.

Role of liver in hypotriglyceridaemic action

Thyroxine displacement. Several mechanisms of action have been proposed to explain how agents like clofibrate bring about reductions in serum triglycerides.

One hypothesis is based on the displacement of thyroxine from its serum-binding proteins by clofibrate resulting in a hyperthyroid effect in the liver and a hypothyroid effect elsewhere. However two agents, novobiocin and trypan blue, which can displace thyroxine from its plasma-binding proteins are incapable of affecting peroxisome populations, catalase activity or hypolipidaemia. These observations, coupled with the finding of a peroxisome/catalase response in thyroidectomized rats treated with clofibrate, lead to the conclusion that thyroxine displacement cannot account for the hypolidaemic action or peroxisome proliferative response of compounds like clofibrate (Masters & Holmes, 1977; Svoboda *et al.* 1969).

Thyroxine displacement by fenofibric acid was weak even at concentrations 100–500 times higher

than those attained in human plasma. In a series of phenoxycarboxylic acids studied, the most active thyroxine displacers were devoid of hypolipidaemic activity and these findings were considered to invalidate the thyroxine-displacement theory (G. F. Blane, unpublished report 1979).

Species differences in peroxisomal involvement and lipoprotein metabolism. A second hypothesis linking catalase levels to hypolipidaemia was discussed earlier, when it was observed that the evidence against this hypothesis outweighed the evidence in its favour.

Masters & Holmes (1977) concluded that hypolipidaemic drugs like clofibrate exert a multivalent effect on intermediary metabolism. The effect on lipid metabolism is mediated via an increased metabolic flux featuring peroxisomal involvement. The hepatic peroxisomal system of β-oxidation of fatty acids appears to play an important role in reducing serum lipids following administration of hypolipidaemic drugs to rodents (Lazarow, 1978).

Important differences in lipoprotein metabolism between the rat and man may also help to explain the difference in hepatic response between the two species. Hypolipidaemic drugs act by increasing the binding of very-low-density lipoproteins (VLDL) and chylomicron remnants to liver cells (Levy, 1979). Rats and man differ markedly in the way they handle lipoprotein remnants bound to liver cells. In rats most of the VLDL and chylomicron remnants are taken up into liver cells whilst in man and primates most of the VLDL and chylomicron remnants are remodelled on the cell surface and are re-exported into the blood as low density lipoprotein (R. H. Hinton, personal communication 1981). Also the effect of fenofibrate on LDL-cholesterol and HDL-cholesterol appears to differ between rat and man (Kritchevsky, 1980b; Rössner & Örö, 1981).

Peroxisomal proliferation as an aetiological factor in liver cancer in rodents

Various hypolipidaemic compounds that cause hepatic peroxisome proliferation in rats or mice produce liver carcinomas when tested in these species. These include clofibrate, Wy-14,643, nafenopin, BR-931 and tibric acid (Table 6) and phthalate esters. Mainly as a result of these observations and the fact that these compounds are chemically unrelated, Reddy *et al.* (1980) stated that the so-called hypolipidaemic peroxisome proliferators form a unique class of chemical carcinogens.

The results obtained so far reveal a remarkably close association between hypolipidaemic peroxisome proliferation and liver-cancer development in rodents. But we consider that peroxisome proliferators do not constitute a novel group of liver carcinogens since they share many common features with another group of hepatocarcinogens possessing microsomal-enzyme inducing properties (see also Tables 7 and 8).

A further indication of the importance of peroxisome proliferation in liver-cancer development in rodents is the finding that at sufficiently low doses, when peroxisome proliferation is absent or minimal (S. C. Price *et al.* unpublished observations 1981), liver tumours do not ultimately appear (Blane & Pinaroli, 1980). However the apparent absence of both peroxisome proliferation and hepatocarcinogenicity at low doses and their combined presence at higher doses needs to be confirmed for hypolipidaemic compounds, especially as at present there is a paucity of dose-response carcinogenicity studies in rodents to make comparisons possible. If this correlation can be confirmed it will offer a remarkable parallel with other hepatocarcinogens such as Ponceau MX and safrole, for which compounds liver-

Table 7. *Classification of rodent hepatocarcinogens according to their patterns of response*

Feature	Class I—classical (nitrosamines, aflatoxin, 4-aminobiphenyl, cycasin)	Class IIa—necrosis-inducing (CCl_4, $CHCl_3$)	Class IIb—microsomal-enzyme inducers (dieldrin, DDT, Ponceau MX, safrole) and peroxisome proliferators (hypolipidaemic drugs)
Potency	*High*—tumours at ppb level	*Low*—tumours at ppm level	*Low*—tumours at ppm level
Versatility	*High*—different types of liver tumours; wide variety of primary tumours at extrahepatic sites	*Low*—hepatic tumours confined to liver cells; wide range of extrahepatic tumours absent	*Low*—hepatic tumours confined to liver cells; wide range of extrahepatic tumours absent
Dose-response	*Wide*—progressive increase in tumour incidence over wide range of dose levels	*Narrow*—sharp increase over narrow range of dose levels	*Narrow*—sharp increase over narrow range of dose levels
Mutagenicity	*Positive*—reaction with DNA, protein, etc.	*Negative*—no reaction with DNA	*Negative*—no reaction with DNA
Liver enlargement	*Absent*	*Absent*	*Present*
Early hepatic response	*Absent*—liver carcinomas can appear without demonstrable early liver damage	*Present*—early liver necrosis	*Present*—early subcellular liver damage; necrosis absent
Liver nodule formation	*Absent*—nodule formation not necessary for liver tumour development (especially at low doses)	*Present*—necessary for liver tumour development	*Present*—necessary for liver tumour development

Table 8. *Role of adaptive response in rodent hepatocarcinogenesis elicited by microsomal-enzyme inducers and hypolipidaemic peroxisome proliferators*

Response	Microsomal-enzyme inducers	Hypolipidaemic peroxisome proliferators
	Adaptive phase	
Liver enlargement	Present	Present
Smooth-endoplasmic-reticulum proliferation	Present	Present
Microsomal-enzyme induction	Present	Absent or transient
Peroxisome proliferation	Absent	Present*.
Initial response reversed after cessation of treatment	Present	Present
Overt early histological damage	Absent	Absent
Liver nodules	Absent	(Absent?)†
Liver tumours	Absent	(Absent?)†
	Consequences of loss of adaptive response	
Liver enlargement	Sustained	Sustained
Smooth-endoplasmic-reticulum proliferation	Sustained	Sustained
Microsomal-enzyme induction	Lost	Absent
Peroxisomal response	Absent	Sustained‡
Early subcellular liver damage progressing to histological liver damage	Present	Present
Liver nodules	Present	Present
Liver tumours	Present	Present

*Elevated catalase and elevated H_2O_2-generating enzymes in a state of homeostasis.
†Awaits experimental confirmation.
‡Relatively small increase in catalase cannot cope with excess H_2O_2 (or other reactive species) generated by prolific rise in H_2O_2-generating enzymes.

cancer development has been shown to occur only at dose levels high enough to elicit an adequate initial hepatic response (Grasso, 1979).

Perhaps the most convincing argument for incriminating peroxisomal involvement in liver neoplasia in rodents lies in the differential effects of hypolipidaemic drugs on peroxisomal enzymes. We have referred earlier to the striking increase in peroxisomal enzymes involved in fatty-acid oxidation (Table 5), during which process H_2O_2 is generated. Whilst urate oxidase, certain oxidases (e.g. D-amino acid oxidase and L-α-hydroxyacid oxidase) and even catalase show at best only minor increases in activity, there are striking increases in the activity of the peroxisomal enzymes that are involved in fatty-acid oxidation and that take on the burden of disposing of the increased load of hepatic lipids resulting from hypotriglyceridaemic action.

Whilst peroxisomal catalase would be expected to cope with slight increases in H_2O_2 levels, high doses of hypolipidaemic drugs in rodents probably cause a breakdown of homeostasis and the excess H_2O_2 generated is unlikely to be degraded in full by the insufficient supply of peroxisomal catalase. Hence liver cells would be exposed to the DNA-damaging potential of H_2O_2. In this connection it is interesting to note that aminotriazole, a potent inhibitor of catalase, produced an increased liver-tumour incidence in acatalasemic mice, a finding which can also be attributed to the impaired degradation of H_2O_2 (Reddy *et al.* 1979). Injurious concentrations of H_2O_2 or other reactive species formed could induce neoplasia either by a direct action on the genome or alternatively *via* a mechanism involving chronic hepatocyte damage leading to reactive nodular hyperplasia.

Comparison of hypolipidaemic drugs with other rodent hepatocarcinogens

General. It is possible to distinguish known hepatocarcinogens into three broad classes according to the features they exhibit, which include the initial hepatic response (Table 7). It will become apparent from the ensuing discussion that hypolipidaemic drugs share many common features with one of these classes, namely those microsomal-enzyme inducing compounds known to be hepatocarcinogenic in rodents (Tables 7 & 8).

Potency, versatility and dose-response. The classical carcinogens exhibit high potency and high versatility in contrast to the other classes of hepatocarcinogens shown in Table 7.

The group of classical carcinogens includes the nitrosamines, aflatoxins, 4-aminobiphenyl, cycasin, vinyl chloride and 2-acetamidofluorene. They induce liver tumours at low levels, often as low as ppb ($b = 10^9$) in the diet, they produce a clear dose-response relationship over a wide range of doses (Druckrey & Schmähl, 1962) and they induce tumours that vary in degree of malignancy from well differentiated to anaplastic hepatocellular carcinomas (Searle, 1976). Furthermore, many of these compounds cause malignant tumours not only in the hepatic parenchymal cells but also in other component cells of the liver, e.g. cells of the vascular epithelium, of the bile ducts, of the Kupffer cells and occasionally of the fibroblasts that form the supporting framework of the liver. Most of these compounds induce tumours in other organs as well. In contrast, hepatocarcinogens in the other classes induce tumours at dietary levels usually in the region of 500–1000 ppm, and only very occasionally

at levels as low as 10 ppm, such as is the case with dieldrin (Walker, Thorpe & Stevenson, 1973). The tumorigenic response is not clearly related to dose or is so only over a limited range of dose levels (Terracini, Testa, Cabral & Day, 1973; Tomatis, Turusov, Day & Charles, 1972) and the tumours induced are always well-differentiated tumours of hepatic-cell origin. Furthermore, no other types of liver tumours are induced and tumours do not appear at other sites.

Hypolipidaemic drugs behave like the microsomal-enzyme inducer class of hepatocarcinogens (Table 7) in respect of low potency and low versatility as well as of a narrow dose-response based on the limited evidence obtained with the hypolipidaemic phthalate esters (National Cancer Institute, 1980 & 1981).

Mutagenicity. Biochemical studies on the classical carcinogens reveal that they react readily, through the agency of active intermediates, with all components of the cell, particularly with protein and DNA, forming covalent-like linkages. The consequences of the binding with protein are not known with certainty but it is widely believed that interaction with DNA leads to mutagenic changes, one expression of which could be the development of cancer (Lutz, 1979).

Compounds in the microsomal-enzyme inducer class of hepatocarcinogens have been studied less extensively but none of the compounds tested exhibited any binding ability with protein or DNA and all have proved negative in at least one mutagenicity test (Bruce & Heddle, 1979; McCann, Choi, Yamasaki & Ames, 1975; Purchase, Longstaff, Ashby, Styles, Anderson, Lefevre & Westwood, 1978).

Negative results have similarly been achieved with hypolipidaemic drugs—fenofibrate/fenofibric acid in a wide range of mutagenicity assay systems (Blane & Pinaroli, 1980; von Däniken *et al.* 1981) and other hypolipidaemic peroxisome proliferators when tested in the Salmonella/microsome assay or in the mouse lymphocyte [^{3}H]thymidine-incorporation assay (Warren *et al.* 1980). These findings prompted the suggestion that hypolipidaemic compounds exert their hepatocarcinogenic action in rats or mice *via* a non-genetic mechanism (Reddy & Qureshi, 1979) similar to the epigenetic mechanism postulated for saccharin (Ashby, Styles, Anderson & Paton, 1978). Even if a genotoxic mechanism is involved, it would not be the result of a primary action of the compound but would probably be secondary to a profound disturbance in intermediary metabolism leading to the formation of a DNA-damaging reactive species in rodent liver.

Liver enlargement. Liver-tumour induction by the classical hepatocarcinogens can occur in the absence of liver enlargement (Kunz, Schaude, Schimassek, Schmid & Siess, 1966). However, liver enlargement is an important feature in hepatocarcinogenesis associated with microsomal-enzyme inducers and hypolipidaemic peroxisome proliferators (Tables 7 & 8).

Studies into the mechanism of liver enlargement are therefore of particular interest. Compounds like phenobarbital, butylated hydroxytoluene and most of its analogues and various chlorinated hydrocarbons produce liver enlargement without histological change. [^{3}H]Thymidine-uptake studies (used to indicate enhanced activity of cell division) conducted on phenobarbital and benzene hexachloride showed that a wave of ^{3}H uptake preceded a wave of mitosis—both events occurring after the first 2–3 daily doses (Schulte-Hermann, 1974).

As mentioned earlier, liver enlargement induced by clofibrate is due to hypertrophy and that induced by nafenopin to a combination of hypertrophy and hyperplasia. Wy-14,643 induces liver cells to proliferate, as evidenced by [^{3}H]thymidine autoradiography and analysis of colchicine-arrested metaphases after a 5-day treatment period (Reddy *et al.* 1979). Increased [^{3}H]thymidine uptake was also evident 24 hr after a single oral dose of Wy-14,643. The absence of hepatocellular necrosis during the first week of Wy-14,643 feeding indicated that the wave of DNA replication and mitosis does not represent reparative hyperplasia, and thus the mitogenic effect is a primary effect of Wy-14,643 (Reddy *et al.* 1979).

The observations with Wy-14,643 are therefore not unique and raise the possibility that this compound and other hypolipidaemic drugs/agents produce liver enlargement in the same way as do phenobarbital and other microsomal-enzyme inducers.

The fact that Wy-14,643, like nafenopin, stimulates DNA replication places these compounds in a class apart from the classical carcinogens, which strongly inhibit DNA replication (Reddy *et al.* 1979).

Early hepatic response, consequences of loss of adaptive response and liver nodule formation in rodents. Hypolipidaemic drugs do not resemble the necrosis-inducing class of hepatocarcinogens (Table 7) because with compounds like carbon tetrachloride and chloroform nodular formation is a consequence of reparative hyperplasia arising from early necrosis (Eschenbrenner & Miller, 1945 & 1946).

The importance of the initial hepatic response in liver-tumour development has been clarified by sequential studies conducted on dieldrin (Hutterer, Klion, Wengraf, Schaffner & Popper, 1969) and on safrole and Ponceau MX (Crampton *et al.* 1977a,b; Grasso & Gray, 1977). Initially all three compounds produced liver enlargement and microsomal-enzyme induction but after a few weeks the level of microsomal-enzyme activity returned to normal despite continuation of treatment. Histological, histochemical and ultrastructural studies of the liver during the period of elevated microsomal enzymes revealed no abnormality apart from an increase in the SER. Once the enzyme induction was lost, mitochondrial changes were observed in the liver of dieldrin-treated mice and lysosomal changes in the liver of rats treated with safrole or Ponceau MX, both changes being indicative of liver damage at the subcellular level. Histological evidence of damage was slow to appear but after several weeks a marked centrilobular fatty change developed in the enlarged liver and this was followed much later by evidence of hyperplastic nodules, some of which progressed into metastasising tumours. Further experiments with Ponceau MX indicated that such changes only take place with dose levels that induce liver enlargement and a biphasic microsomal-enzyme response (Crampton *et al.* 1977a,b; Grasso & Gray, 1977).

This sequence of events, which can be summarized as chronic liver injury → nodular hyperplasia → carcinoma, is very different from that observed with the classical carcinogens. Sequential studies with nitros-

amines, aflatoxin or 4-aminobiphenyl indicate that malignant tumours may arise *de novo* without the intervention of any recognisable phase of histological liver damage and very often without evidence of a phase of nodular hyperplasia (Grasso & Gray, 1977). Williams (1980) agrees that the hyperplastic nodule is not an obligatory precursor of carcinoma and that hyperplastic foci can give rise directly to carcinomas.

The initial hepatic response seen with hypolipidaemic drugs is similar in many respects to that observed with the enzyme-inducing class of hepatocarcinogens (Tables 7 & 8). Both types of compound produce liver enlargement and proliferation of the SER, associated either with microsomal-enzyme induction in the case of the enzyme-inducing type of hepatocarcinogen or with peroxisomal proliferation in the case of the hypolipidaemic drugs. A train of events leading to hepatic neoplasia sets in after the loss of microsomal-enzyme induction which marks the termination of the adaptive phase with compounds like Ponceau MX, safrole and dieldrin. By analogy, in the case of hypolipidaemic drugs, events leading to neoplasia could set in after the breakdown in homeostasis when catalase insufficiency may allow excess peroxide to persist long enough to cause either DNA damage or non-specific hepatic damage leading to hyperplasia and eventually neoplasia.

Extrapolation of rodent findings to man

We have already referred to important differences in the way rat and human livers dispose of lipids following treatment with hypolipidaemic drugs. The presence of peroxisomal involvement in the rat and its absence in man is analogous to the situation with certain microsomal-enzyme inducers for which hepatocarcinogenic action in rodents is not accepted as being relevant to man (WHO, 1979).

In DDT-treated mice, changes in liver progress from hypertrophy, margination and lipospheres in isolated, centrilobular hepatocytes to the formation of nodules of affected cells. The entire continuum of changes induced by phenobarbital, DDT and other organochlorine insecticides from the prompt response in isolated cells to the eventual formation of liver tumours is peculiar to rodents and does not occur in other species in which the endoplasmic reticulum does not respond morphologically in the same way. There is no evidence that anything from the first increase in endoplasmic reticulum to the final development of a highly nodular liver with occasional displacement of cells to the lungs has any bearing on the health of man or other animals in which the endoplasmic reticulum does not respond in this way (WHO, 1979).

From the studies on hypolipidaemic drugs described in this review, there is reasonable evidence to indicate that the morphological response of the endoplasmic reticulum and in particular the peroxisome proliferative response seen in rodents do not feature in the limited studies hitherto conducted in monkeys and man. More conclusive evidence may emerge from further studies in these latter species.

On the basis of the evidence obtained so far with hypolipidaemic drugs and of the greater experience gained with rodent hepatocarcinogenesis from microsomal-enzyme inducers, it is considered unlikely that man would be susceptible to the tumorigenic action of hypolipidaemic drugs as is the rodent.

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INTERSPECIES RESPONSE TO CARCINOGENS AND OESTROGENS

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Summary—Carcinogenic chemicals produce specific malignancies in a wide variety of animal species and in a high proportion of the treated animals. Oestrogens do not demonstrate a carcinogenic profile, producing malignancies in a limited number of animal species and in a low proportion of the treated animals. The effect of oestrogens depends on both species and strain.

Introduction

Dr Leon Golberg has contributed to toxicology not only as the founder and longtime Editor of *Food and Cosmetics Toxicology*, but also by his research and publications (cf. Golberg, 1974, 1978).

It may be remembered that Dr Golberg was one of the discoverers of diethylstilboestrol, the first synthetic oestrogen to be developed (Dodds, Golberg, Lawson & Robinson, 1938). The oestrogens are often cited as being carcinogenic in animal studies, and in this brief review I should like to summarize responses of a range of species to oestrogens and to contrast the results with the effects of carcinogens. The discussion of the effects of oestrogens will be limited to their action on the breast and the uterine cervix and endometrium.

Interspecies response to carcinogens

Compounds such as 2-naphthylamine, aflatoxin B_1, asbestos, and the other examples listed in Table 1, produce their characteristic tumorigenic effect in a number of different species (IARC, 1972, 1974, 1976 & 1977; Irving, Wiseman & Young, 1967; Miller & Enomoto, 1964; Miller, Miller & Hartman, 1961). Clearly the response to a specific carcinogen is not limited to one or two species but, allowing for differences in absorption and metabolism, is a general biological response obtained in a variety of species. Indeed, based on the concept of covalent binding to DNA, we would expect a carcinogen to produce essentially the same effect in different species of animals. When a given compound induces the same carcinogenic response in several different species it may be predicted, depending on dosage and duration of exposure, that a carcinogenic response will also be obtained in man.

Two other characteristics of carcinogens should also be noted:

(i) Carcinogens induce their specific neoplastic effects in a high proportion of the treated animals (Table 2).
(ii) The increased incidence of specific neoplasms is obtained in a relatively short period of time. The neoplastic response is discerned before the end of an 18-month toxicity study in the mouse or a 2-yr chronic toxicity study in the rat.

Interspecies response to oestrogens

The effect of oestrogens on the occurrence of malignancies of the mammary gland, uterine cervix and endometrium in different species of animals is summarized in Table 3. It is apparent from this summary

Table 1. *Examples of species' responses to carcinogens*

Compound*	Target site	Carcinogenic response of							
		Mouse	Rat	Hamster	Guinea-pig	Rabbit	Dog	Monkey	Man
2-Naphthylamine	Bladder	+	0	+ +			+	+	+
4-Aminobiphenyl	Bladder	+	+			+	+		
MNNG	GI tract	+	+	+		?	+		
N-OH-AAF	GI tract	+	+	+	+	0			
Aflatoxin B_1	Liver	+†	+					+	+
Asbestos‡	Pleural mesothelium, lung	+	+	+		+			

\+ = Positive effect 0 = No effect
MNNG = *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine *N*-OH-AAF = *N*-Hydroxy-2-acetylaminofluorene
*Administered orally unless otherwise stated.
†Intraperitoneal; ineffective orally in the mouse.
‡Inhalation.

Table 2. *Time and intensity of carcinogenic response. Effect of* N-*hydroxy-2-acetylaminofluorene in mice and rats*

Target site		Percentage of animals with cancer		
	In female mice* after ...	**10 months**	**12 months**	**14 months**
Mammary gland,	treated	9	19	30†
	control	7·5	12·5	15†
Forestomach‡		2	12	23
Liver‡		5	5	12
Urinary bladder‡		0	2	9
	In rats* after ...	**3 months**	**5 months**	**8 months**
Mammary gland‡	M			0
	F			67
Forestomach‡	M			9
	F			60
Liver‡	M	27	45	64
	F	0	0	13

*Mouse data from Miller *et al.* 1964; rat data from Miller *et al.* 1961.
†Survival rates of the treated and control groups differed; life-table analysis is necessary.
‡Data is for treated animals only since there were no malignant tumours in control mice at 14 months, or in control rats at 8 months.

that oestrogens do not produce comparable effects in several different species or a response of the magnitude that is characteristic of a carcinogen. A positive effect of oestrogen is restricted to certain special situations.

Breast cancer

Articles on hormones and cancer may state that exogenous oestrogen causes breast cancer in mice, but such a statement is only partly true. It is usually not recognized or appreciated that oestrogen will increase the incidence of breast cancer only in *male* mice from inbred strains that possess a transmissible milk factor (a mammary tumour virus); oestrogen is ineffective in male mice from a low-tumour strain, i.e. without the milk factor. Neither mestranol nor ethinyloestradiol increases the occurrence of mammary cancer in CF-LP females, a low-tumour strain (Committee on Safety of Medicines, 1972; Drill, 1974; Lee, 1979).

The effect of oestrogen in the female rat is very variable. As indicated in Table 3 oestrogen may be without effect or may produce mammary cancer in as many as 27% of the treated animals. The reason for this marked variation is unknown although it may possibly represent in part the variation that is encountered in untreated female rats. For example, Durbin *et al.* (1966) found the normal incidence of mammary adenocarcinoma in untreated rats to vary between 0 and 18%, and in our studies the probability of tumour development based on life-table analysis varied from 0 to 15·7% (Drill, 1974).

References to studies of breast cancer in other species are cited in several reviews (Drill, 1975 & 1981). Of particular interest is the absence of an effect of oestrogen in dogs and monkeys treated with different doses of oestrogen for 6 or more years.

A variety of clinical studies also demonstrates the lack of a relationship between exogenous oestrogen and breast cancer. In five retrospective case-controls there was no association between use of an oestrogen and the relative risk for breast cancer, and a series of 12 clinical studies did not demonstrate an increased occurrence of cases of breast cancer in women treated with oestrogens (cf. Drill, 1975 & 1981).

Cervical cancer

The oral administration of oestrogen may occasionally induce cervical carcinomas in mice, but oestrogens do not produce cervical cancer in other species (Drill, 1976). Even in the mouse the overall incidence of cervical carcinomas is low and is not characteristic of the effects of a carcinogen (Table 3). Clinical studies do not provide any evidence that the long-term use of oestrogen induces cervical cancer (Drill, 1976), indicating good agreement between the experimental and clinical data.

Endometrial cancer

The relationship of exogenous oestrogen to endometrial cancer is currently the subject of intensive clinical study. However, oestrogens do not act as typical carcinogens in animals, producing a slight

Table 3. *Comparison of carcinogenic effects of oestrogens in female animals*

Target site	Carcinogenic response to oestrogens of↦						
	Mouse	Rat	Guinea-pig	Rabbit	Dog	Monkey	Man
Breast	0*	+†	0	0	0	0	0
Uterine cervix	±(1·4%)	0	0	0	0	0	0
Endometrium	±(2·1%)	0	0	+?	0	0	0?

0 = No effect ± = Borderline effect + = Positive effect
*Positive in male mice with mammary tumour virus.
†Range from 0% to 27% with adenocarcinoma.

effect in the mouse and possibly a positive effect in the rabbit, but no effect in other species (Table 3). A strain difference in response may be present in mice, as the carcinoma was present in eight of 71 BDH-SPF mice and only in 3 of 461 other mice (Drill, 1979 & 1980). Carcinomas may possibly occur in the rabbit, but information in this species is very limited; the rabbit may be an atypical species as cancer may occur in as many as 56% of untreated rabbits in the Tans breed (Greene, 1959).

A complete review of clinical data on oestrogen administration and endometrial cancer is beyond the scope of this paper and only general comments will be made on the subject. Much of the clinical data is derived from retrospective case-control studies (which measure association), and in a tabulation of nine studies a risk was absent in four studies and present in five of the studies (Drill, 1980). In the study of Horwitz & Feinstein (1978) the relative risk varied from 12·0 to 1·7 depending on the sampling procedure (see also Horwitz & Feinstein, 1980).

More recently, an increase in risk has also been reported by Hulka, Kaufman, Fowler, Grimson & Greenberg (1980) and Shapiro, Kaufman, Slone, Rosenberg, Miettinen *et al.* (1980) and some authors have found a higher risk with increasing duration of use of oestrogens. In contrast, a series of retrospective case-control studies conducted in Europe has failed to demonstrate a risk for endometrial cancer in oestrogen-users (Lauritzen, 1978; Salmi, 1979 & 1980; Völker, Kannengiesser, Majewski & Vasterling, 1978). Antunes, Stolley, Rosenhein, Davies, Tonascia *et al.* (1979) found a relative risk of either 6·0 or 2·1 depending on the control group selected, indicating again the variation in results that can be obtained. Ultimately, prospective clinical data will be required if we are to reach a definite conclusion on oestrogens and endometrial cancer, and in view of the present status of this area the clinical studies are indicated by a question mark in Table 3.

Discussion

It has been established that chemicals that are carcinogenic in man will produce a similar cancer in a variety of animal species. In view of this correlation, chemicals and drugs can be evaluated in animals to predict potential human carcinogenicity. The suspected carcinogen should produce an effect that is not limited to one (or two) species of animals (Table 1); indeed, based on a DNA mechanism we would expect to obtain a similar organ response in most of the species tested. Secondly, in appraising carcinogenicity the magnitude of the response should also be considered; it is usually not appreciated that the known carcinogens produce a positive organ-specific response in a high proportion of the test animals (Table 2).

Research papers on hormones often state that oestrogens have been shown to be carcinogenic in animals, that oestrogens induce tumours in five species of animals, or that oestrogens are hormonal carcinogens. Although such statements have some validity when applied to specific animal studies, they are in general based on a lack of understanding of the effects of oestrogens in animals. For example, it is often stated that oestrogens induce cancer in mice, but it is not stated or appreciated that the effect is confined to male mice from specific inbred strains that possess a mammary tumour virus (Drill, 1981). The effect of oestrogens on the mammary gland, uterine cervix or the uterine endometrium of different animal species does not demonstrate a carcinogenic profile. Although oestrogens may produce an organ-specific response in the mouse or rat (Table 3), they do not produce the interspecies effect characteristic of a carcinogen. The effect of oestrogen is strain- and species-dependent. Also, when a positive response is obtained only a small proportion of the treated animals are affected, a response that is not typical of a carcinogen.

Clinical data do not demonstrate a relationship between oestrogen administration and the occurrence of breast or cervical cancer, agreeing well with the absence of a carcinogenic profile in animal studies. The clinical appraisal of oestrogens and endometrial cancer is still in progress and in view of the conflicting data that have been obtained, a definite conclusion cannot presently be drawn.

It is generally accepted that the induction of cancer by chemicals involves the covalent binding of the chemical or its metabolites to DNA. Thus, an estimate of carcinogenic potential can be made by procedures that are not dependent on tumour induction. In a highly significant study Lutz & Schlatter (1979) measured the covalent binding of various chemicals to DNA in rat liver. They demonstrated that very potent hepatocarcinogens such as aflatoxin B_1 or M_1 have a high covalent binding index (CBI). Benzene, which does not induce liver tumours gave a very low CBI, and oestrone and ethinyloestradiol gave a CBI slightly less than that of benzene, essentially a negative result.

Oestrogens are necessary for the growth and function of the female reproductive organs and to the extent that they produce functional organs in women they lay the groundwork for the development of cancer. As such they are not carcinogens, but they develop the organs to a functional state that is responsive to carcinogenic influences. The oestrogens have at times been called cocarcinogens or facultative carcinogens, but such terms are not entirely satisfactory; perhaps the oestrogens are best described as having a *non-specific permissive action.*

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STUDIES ON THE MECHANISM OF CARCINOGENICITY OF DIETHYLSTILBOESTROL: ROLE OF METABOLIC ACTIVATION

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Summary—The oxidative metabolism of oestrogenic diethylstilboestrol, hexoestrol, and dienoestrol is reviewed briefly. Diethylstilboestrol and some of its metabolites have been shown to be capable of causing genetic damage in several *in vitro* systems. This raises the possibility that a genotoxic mechanism may contribute to the transplacental carcinogenicity of this synthetic oestrogen, and the possible role of metabolic activation in the carcinogenic effect is considered.

Introduction

More than 40 years ago a group of English scientists, including Leon Golberg, undertook an intensive search for nonsteroidal compounds with oestrogenic activity, in order to obtain an inexpensive oestrogen. Their efforts were highly successful in 1938, when a series of 1,2-diphenylethane derivatives was synthesized (Dodds, Golberg, Lawson & Robinson, 1938 & 1939). Three compounds, namely diethylstilboestrol (DES), α-dienoestrol (E,E-DIES), and *meso*-hexoestrol (HES) proved to be the most oestrogenic substances in that series. The structures of these compounds are shown in Fig. 1. Apart from their inexpensive synthesis, the stilbene-type oestrogens had the therapeutic advantage of being more effective after oral administration than the natural steroid-type oestrogens, oestradiol-17β and oestrone. Therefore it is not surprising that they were enthusiastically welcomed by many clinicians and were already in clinical use in America and Europe by late 1938 (Dodds, 1962).

Use of the stilbene oestrogens was indicated primarily in cases of oestrogen deficiency (as hormone substitutes) and in the treatment of prostatic tumours. Following reports that DES might prevent miscarriages, its therapeutic use in threatened abortion became popular, leading to its administration to an estimated 2–4 million pregnant women in the USA (Fink, 1978). Moreover, DES proved to be effective in fattening chickens and promoting the growth of beef cattle and sheep. This led to its extensive use in the cattle industry. It has been estimated that prior to the recent ban by the FDA, more than 80% of the cattle raised in the USA received DES (McLachlan & Dixon, 1976).

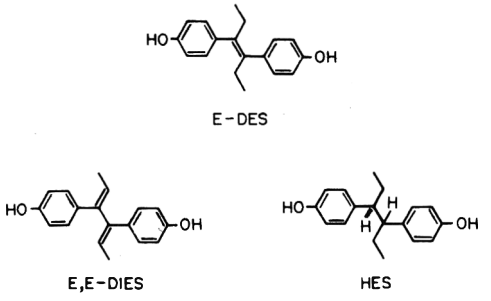

Fig. 1. Chemical structure of diethylstilboestrol (DES), dienoestrol (DIES) and hexoestrol (HES). E (and Z, see Fig. 2) are configurational descriptors denoting the isomerism at the olefinic double bond.

Although there were several reports on the tumorigenicity of DES in various animal species as early as 1938, as shown in reviews by McLachlan & Dixon (1976) and McMartin, Kennedy, Greenspan, Alam, Greiner & Yam (1978), the potential health hazard posed by DES was only recognized following the publication, from 1971 onwards, of reports by Herbst and his associates, linking certain malignant tumours and non-malignant abnormalities of the genital tract in young women with *in utero* exposure to DES (Herbst, 1976; Herbst, Ulfelder & Poskanzer, 1971). The transplacental carcinogenicity and teratogenicity of DES suggested by these findings were confirmed by animal experiments (IARC Working Group, 1979) and finally led to the abandonment of DES for most therapeutic and agricultural purposes.

One of the questions arising from the foetotoxic effects of DES concerns the mechanism of this toxicity, and particularly of the compound's carcinogenicity. Is the carcinogenicity due to the hormonal activity of DES, or is DES acting as a chemical carcinogen, or are both mechanisms operative? The mechanism(s) by which hormones result in cancer development is not understood but is assumed to differ basically from that for chemical carcinogens. For the latter, electrophilic reactivity of the carcinogen or of a metabolite is a prerequisite, and the tumorigenic event appears to be initiated by covalent binding to critical cellular macromolecules, presumably DNA. Hormonal carcinogens, on the other hand, are considered to lack chemical reactivity and, consequently, are not able to bind covalently to DNA.

A first step in evaluating the role of metabolism in the mechanism of DES carcinogenicity is to demonstrate that reactive metabolites are formed and that

they are able to cause genetic damage. The present state of knowledge in this area is summarized briefly in this paper. Recent data on the metabolism of HES and E,E-DIES are also presented. As will be shown, there are significant differences in the metabolism of these three stilbene oestrogens. This fact may have a bearing on their carcinogenicity and may aid in the identification of the metabolic pathway(s) critical for the carcinogenic effect of DES.

The metabolism of diethylstilboestrol (DES)

Despite the widespread use of DES, not much attention was paid to its metabolism until recently. It was observed at an early date that DES is easily conjugated with glucuronic acid (Mazur & Shorr, 1942) and, until 1975, the DES monoglucuronide was the only established metabolite. From our investigations with aminostilbene derivatives (Metzler & Neumann, 1977), we predicted that oxidative metabolism could also be expected in the case of DES (Metzler, 1975), and we accordingly initiated fairly extensive studies of the metabolic fate of DES *in vivo* in several species including man and non-human primates. These studies have been reviewed in detail by Metzler (1981). Independently but at the same time, the oxidative metabolism of DES in rat liver was studied *in vitro* (Engel, Weidenfeld & Merriam, 1976). Both studies indicated that DES is prone to oxidative metabolism, and several new metabolites were subsequently identified. These are depicted with their putative metabolic intermediates in Fig. 2.

From this metabolic scheme, it is clear that virtually every part of the DES molecule is affected by metabolic oxidation. Thus, aromatic and aliphatic hydroxylations and epoxidation of the olefinic double bond occur, presumably mediated by cytochrome *P*-450-dependent mono-oxygenases. Of particular interest was the finding that the stilbenediol structure can be oxidized to Z,Z-dienoestrol (Z,Z-DIES) and that this reaction is mediated by peroxidases (Metzler & McLachlan, 1978b).

Oxidative metabolites are not confined to adult organisms, but have also been found in foetal and neonatal mice (Metzler & McLachlan, 1978c) and in foetal hamsters (Gottschlich & Metzler, 1981). More recent results indicate that in the mouse even the ultimate target organ for the foetotoxic effect of DES, the foetal genital tract, can oxidize DES (Maydl, Newbold, Metzler & McLachlan, 1981).

Reactive metabolites and genetic toxicity of DES

From the structures of some of the DES metabolites and intermediates (Fig. 2), electrophilic reactivity can be anticipated. Candidates include the arene oxide precursor of 3'-hydroxy-DES, and products possibly derived from that catechol, e.g. an *o*-semiquinone and -quinone. Other potential electrophiles are the DES-4',4''-semiquinone and -quinone, and the DES-3,4-oxide. Finally, there are allylic alcohols (1-hydroxy-Z,Z-DIES and 1-hydroxy-ψ-DES), which upon esterification give rise to alkylating compounds (Metzler, 1978).

Evidence supporting the formation of reactive species in DES metabolism has been provided by the non-extractable binding to tissue macromolecules observed in numerous *in vivo* and *in vitro* metabolic studies in which radioactively labelled DES was used. These have been reviewed by Metzler, Gottschlich & McLachlan (1980). Eventually it was possible to show that DES binds covalently to DNA both *in vivo* (Lutz,

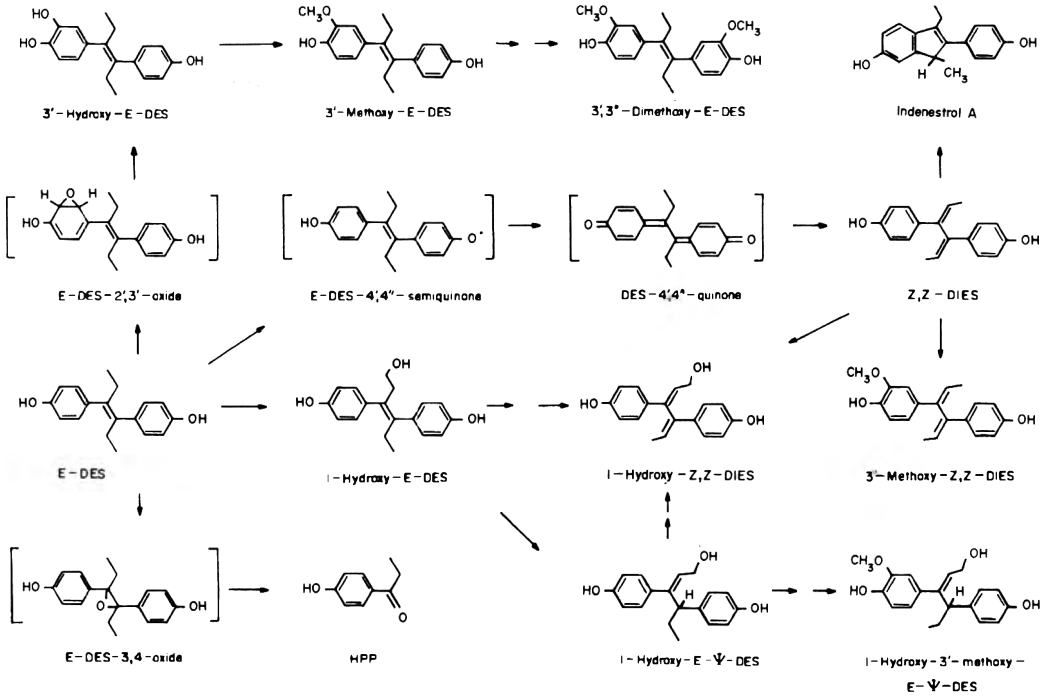

Fig. 2. Oxidative pathways in the metabolism of diethylstilboestrol (DES). Compounds in brackets are putative metabolic intermediates. DIES—dienoestrol; nomenclature according to Metzler & McLachlan (1978a).

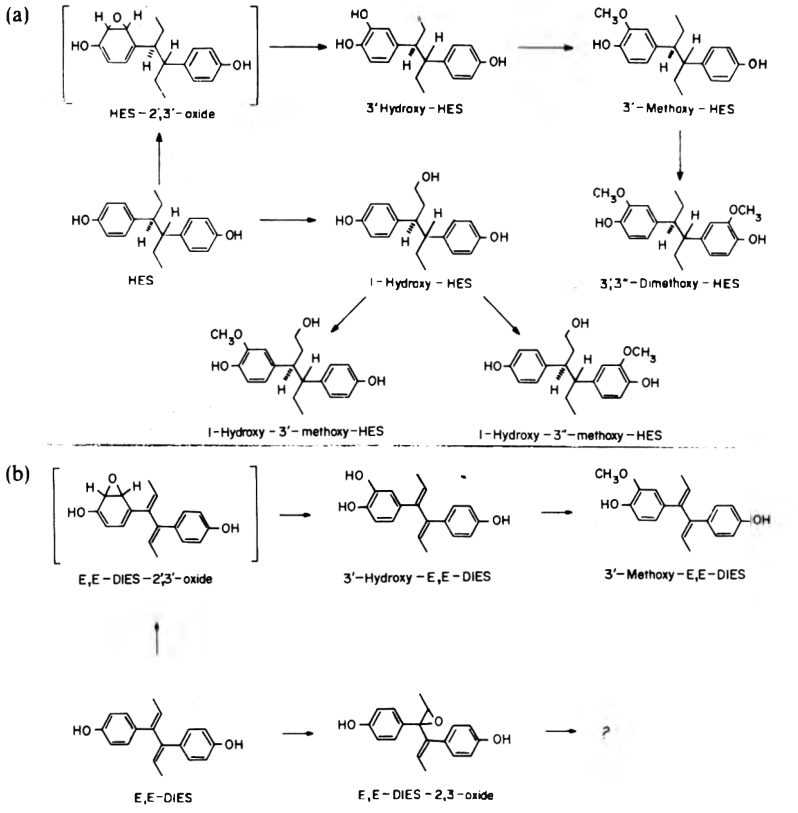

Fig. 3. Oxidative pathways in the metabolism of (a) *meso*-hexoestrol (HES) and (b) E,E-dienoestrol (E,E-DIES).

1979) and *in vitro* under various metabolic conditions, including incubation with rat hepatic microsomes (Blackburn, Thompson & King, 1976), mouse hepatic and uterine microsomes (Okey & Nebert, 1979) and mouse-uterus peroxidase preparations (Metzler & McLachlan, 1978b). Moreover, several short-term tests designed to assay the genetic toxicity of chemical carcinogens have recently shown a positive response to DES. These tests were a skin test in hairless mice (Forsberg, 1979) and tests based on induction of unscheduled DNA synthesis in HeLa cells in the presence of microsomes (Martin, McDermid & Garner, 1978), mutations in a mouse lymphoma cell line (Clive, Johnson, Spector, Batson & Brown, 1979), sister chromatid exchange (SCE) in human fibroblasts (Rüdiger, Haenisch, Metzler, Oesch & Glatt, 1979), chemical transformation of embryo-derived mouse fibroblast cells (Purdy, Meltz, Goodwin & Williams, 1979) and chemical transformation of Syrian hamster embryo cells (Pienta, 1980). In the SCE assay, the activity of DES could be completely suppressed by α-naphthoflavone, an inhibitor of drug metabolism. In this test, DES-3,4-oxide and Z,Z-DIES, two metabolites of DES, were more active than DES by factors of 10 and 70, respectively, suggesting that the genetic toxicity of DES is, in fact, due to metabolic activation (Rüdiger *et al.* 1979).

It remains to be established, however, which of the several reactive DES metabolites indicated above accounts for the macromolecular binding and genetic toxicity. One approach that should answer this question and further clarify the role of metabolic activation in the carcinogenicity of stilbene oestrogens is the study of the metabolism and genetic toxicity of DES analogues which retain their oestrogenicity but have certain metabolic pathways blocked. Two such compounds are HES and E,E-DIES (Fig. 1).

Metabolism of *meso*-hexoestrol (HES)

The oxidative biotransformation of HES has been studied by analysing the glucuronide fractions from rat bile and mouse urine (Metzler & McLachlan, 1981). At least six metabolites were identified from their mass spectra and comparison with reference compounds. The metabolic scheme proposed for HES is given in Fig. 3a. As might be expected, metabolic oxidations are restricted to the aromatic and aliphatic portions of the molecule. Other activating pathways that are possible in DES metabolism, namely formation of an olefinic epoxide and a *p*-quinoid compound, are not feasible in HES metabolism because of the lack of the stilbene double bond.

HES is as powerful an oestrogen as DES (Dodds *et al.* 1939). There appear to be no data in the literature about its transplacental toxicity or its genetic toxicity in short-term assays.

Metabolism of E,E-dienoestrol (E,E-DIES)

When the glucuronide fraction from rat bile sampled after administration of E,E-DIES was analysed by gas chromatography/mass spectrometry, the ring-hydroxy and methoxy derivatives of E,E-DIES were identified (Fig. 3b). In addition, however, there was clear evidence for the presence of an olefinic epoxide

(Metzler & McLachlan, 1981). Both the epoxide itself and two rearrangement products could be seen in the gas chromatogram, in a pattern which was also obtained when synthetic DIES-2,3-oxide was gas-chromatographed. Therefore, DIES must be considered to be oxidized at the aromatic ring and at the olefinic double bond. There was no indication of aliphatic hydroxylation.

The oestrogenicity of E,E-DIES is about the same as that of E-DES (Dodds *et al.* 1939). Again, no information is available so far about the foetotoxicity and genotoxicity of this compound.

Conclusion

Current evidence shows that the synthetic oestrogen, DES, is metabolized to a variety of oxidative metabolites along several pathways involving reactive intermediates. This fact, and a positive response in several assays indicating genetic damage, raise the possibility that metabolic activation may be involved in the carcinogenic effect of DES. The exact mechanism(s) accounting for the organ-directed carcinogenicity of DES is not known, but several possibilities are conceivable (Metzler & McLachlan, 1979). Examples are the formation of reactive metabolites that retain their oestrogenicity and are thus accumulated in oestrogen target organs, and the preferential formation of reactive metabolites in those organs by specific enzymes. The first possibility can be exemplified by E-DES-3,4-oxide (Fig. 2), which has been found to be highly oestrogenic *in vivo* and *in vitro* (Korach, Metzler & McLachlan, 1978). An attractive hypothesis for the organ-specific activation of DES would be peroxidase-mediated oxidation, since oestrogen-inducible peroxidase activity exists in all organs that depend on oestrogens for growth (Anderson, Kang & DeSombre, 1975).

It appears that certain DES analogues such as HES and E,E-DIES may be useful for clarifying further the role of metabolic activation in the genetic toxicity and carcinogenicity of DES. These compounds are powerful oestrogens but they have different routes of metabolism. It will be of interest to determine whether their effects in the different *in vitro* and *in vivo* systems can reasonably be correlated with certain metabolic pathways.

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STRUCTURE–MUTAGENICITY ANALYSIS WITH THE CHO/HGPRT SYSTEM*

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Summary—Using a mammalian cell gene mutational assay, the Chinese hamster ovary cell/hypoxanthine–guanine phosphoribosyl transferase (CHO/HGPRT) system, we have studied the structure–mutagenicity of ten alkylating agents and six platinum(II)chlorammines. In analysing the mutagenesis data, we describe the mutagenic activity as the number of mutants per 10^6 clonable cells induced by a 1 μM concentration of chemical tested. The mutagenicity of alkylating chemicals decreases with increasing size of the alkyl group; methylating agents are three to six times more mutagenic than the corresponding ethylating agents. *cis*-$Pt(NH_3)_2Cl_2$ is mutagenic, but its steric isomer, *trans*-$Pt(NH_3)_2Cl_2$, is very much less mutagenic. These results, together with determination of chemically induced DNA lesions permit analyses of certain aspects of mechanisms of chemical mutagenesis.

Introduction

Advances in the study of the genetics of mammalian somatic cells in culture have contributed much to the recent rapid progress in experimental biology and medicine. This article demonstrates the role that somatic-cell genetics has played in our gradual appreciation of the genetic aspects of chemical toxicology. The use of ten alkylating chemicals and six platinum(II) compounds for analysis of the effect of structure modification on chemical mutagenicity reflects a progression from studying pure model mutagens to examining antitumour agents used clinically.

Gene mutation in mammalian cells

Since the first demonstration that variants with altered nutritional requirements and drug sensitivity could be induced after treatment with a chemical mutagen in two near-diploid mammalian cell lines, CHO (Puck & Kao, 1967) and V79 (Chu & Malling, 1968), there has been interest in utilizing these cells for studying mechanisms of mammalian genetics. Many variants with a wide spectrum of phenotypes have since been isolated and characterized from these and other cells (Siminovitch, 1976; Thompson & Baker, 1973).

This article describes the use of a mutational system which affects the hypoxanthine–guanine phosphoribosyl transferase (HGPRT) activity in Chinese hamster ovary (CHO) cells, the CHO/HGPRT system (Hsie, Brimer, Mitchell & Gosslee, 1975a; Hsie, O'Neill, Couch, San Sebastian, Brimer *et al.* 1978; O'Neill, Brimer, Machanoff, Hirsch & Hsie, 1977), to illustrate aspects of gene mutation and its subsequent utilization in a quantitative assessment of structure–mutagenicity. To study the genetic, biochemical and molecular bases of gene mutation, one needs only to isolate one or a few mutants of the desired phenotypes; however, studies of quantitative mutagenesis require that a mutational protocol selects for mutants, a great majority (or all) of which are affected at a single gene. For mutagen screening a quantitative single-locus mutational assay must also have intra- and interlaboratory reproducibility (Hsie, 1980; Hsie *et al.* 1978).

The CHO/HGPRT system

We used CHO-K_1-BH_4 cells (Hsie *et al.* 1975a), a subclone of the near-diploid CHO cell line, for this study. CHO cells are genetically well characterized, grow well either on solid substrates or in suspension, with a population doubling time of 12–14 hr, and exhibit a high cloning efficiency and a relatively stable karyotype. They have been used extensively for studying mutagen-induced cytotoxicity and gene mutation and have been favoured for cytogenetic studies.

Using a previously standardized experimental procedure for culturing the cells, treatment with chemicals, and measurement of cytotoxicity and gene mutation (Hsie *et al.* 1975a; Hsie *et al.* 1978; O'Neill *et al.* 1977), we measured gene mutation by quantifying the frequency of mutants resistant to a purine analogue, 6-thioguanine (TG). A metabolic activation system derived from Aroclor 1254-induced male Sprague–Dawley rat livers was used to determine the mutagenicity of promutagens (Hsie, 1980; Machanoff, O'Neill & Hsie, 1981; O'Neill *et al.* 1977). For data presentation, we termed cytotoxicity as the effect of chemical treatment on the cloning efficiency of cells relative to the untreated control, which is routinely 80% or higher; mutagenicity was expressed as the number of TG-resistant mutants per 10^6 clonable cells; the spontaneous mutation frequency generally lies at 0–15 $\times$ 10^{-6} mutants/cell.

*Joining the list of many, I am writing this article to honour the distinguished contribution of Dr Leon Golberg to toxicology, the journal *Food and Cosmetics Toxicology* and the British Industrial Biological Research Association.

Questions concerning the genetic *versus* epigenetic origin of phenotypic variants have been raised earlier for studies of mutagenesis with mammalian cells. In the absence of direct evidence of gene mutation through analyses of the nucleotide sequence of the *hgprt* gene and the amino-acid sequence of the HGPRT protein for TG-resistant mutants, we have exhaustively accumulated genetic, biochemical, and physiological evidence that indicates that the CHO/HGPRT system fulfils the criteria for a specific-locus mutational assay (Hsie *et al.* 1978).

Quantitative mutagenesis with the CHO/HGPRT system

In our early studies we demonstrated that mutation induction by a direct-acting chemical mutagen, ethyl methanesulphonate (Hsie *et al.* 1975a), and a physical agent, ultraviolet light (Hsie, Brimer, Mitchell & Gosslee, 1974b), was quantifiable. Treatment of CHO cells with EMS (25–800 μg/ml) for 16 hr caused a concentration-dependent exponential killing after a distinct shoulder region where there was no appreciable loss of cell survival. However, mutation induction occurred over the entire concentration range tested (Fig. 1; Hsie *et al.* 1975a). Further studies using treatment times of 2–24 hr and various EMS concentrations demonstrated the existence of a limited reciprocity of EMS mutagenesis; that is, when different EMS concentrations (0·05–3·2 mg/ml) were multiplied by various treatment times (2–10 hr), the product [(mg/ml) × hr] yielded a constant mutation frequency and cytotoxicity (O'Neill & Hsie, 1977).

The quantitative nature of the CHO/HGPRT system has been used for studies of the structure–mutagenicity of ten alkylating chemicals: dimethyl sulphate (DMS), diethyl sulphate (DES), methyl methanesulphonate (MMS), ethyl methanesulphonate (EMS), isopropyl methanesulphonate (iPMS), *N*-nitroso-*N*-methylurea (MNU), *N*-nitroso-*N*-ethylurea (ENU), *N*-nitroso-*N*-butylurea (BNU), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), and *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG). Six platinum(II) compounds have also been investigated: *cis*-dichlorodiammine platinum(II) (*cis*-$Pt(NH_3)_2Cl_2$), *trans*-dichlorodiammine platinum(II) (*trans*-$Pt(NH_3)_2Cl_2$), potassium trichlorammineplatinate(II) ($K[Pt(NH_3)Cl_3]$), chlorotriammine platinum(II) chloride ($[Pt(NH_3)_3Cl]Cl$), potassium tetrachloroplatinate(II) [$K_2(PtCl_4)$], and tetraammine platinum(II) chloride ($[Pt(NH_3)_4]Cl_2$). All ten alkylating agents are carcinogenic in laboratory animals (IARC Working Group, 1974a,b & 1978; Schoental & Bensted, 1969; Ward & Weisburger, 1975), and of the platinum(II) compounds *cis*-$Pt(NH_3)_2Cl_2$, is an inorganic antitumour agent which is particularly effective in combination therapy against testicular and ovarian cancer (Einhorn & Donahue, 1977; Wittshaw & Kroner, 1976).

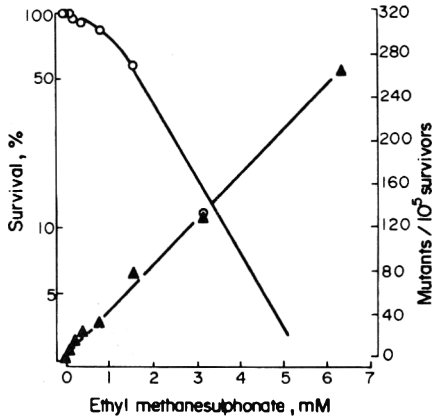

Fig. 1. Dose–response relationship of cytotoxicity (○) and mutagenicity (▲) of ethyl methanesulphonate. The average cloning efficiency of the untreated control culture is 80%, and the spontaneous mutation frequency is 12×10^{-6} mutants/clonable cell. [Redrawn from Hsie, Brimer, Mitchell and Gosslee (1975a).]

Alkylsulphates and alkanesulphonates

All alkylsulphates and alkanesulphonates have been found to be cytotoxic and mutagenic to CHO cells. Despite the vast difference in the size of the shoulder region and the slope of the survival curve, these alkylating agents have been shown to induce mutation with a linear increase over the entire survival curve (Couch, Forbes & Hsie, 1978; Couch & Hsie, 1978; Hsie *et al.* 1975a). The data in Table 1 show that the alkylsulphates vary considerably in cytotoxicity; for DMS and DES concentrations of 90 and 2800 μM, respectively, are required to reduce cell survival to 10%. At equitoxicity, DES is more mutagenic than DMS. Since DES is much less cytotoxic than DMS, DES is one-third as mutagenic as DMS when the comparison is made on a molar basis (Table 1).

Cytotoxicity has also been found to decrease with an increase in the size of the alkylating substituent among alkanesulphonates (Table 1); concentrations of 95, 4540 and 3700 μM MMS, EMS and iPMS, respectively, are required to reduce cell survival to 10%. MMS induces four- and 15-fold more mutations than EMS and iPMS, respectively, on a molar basis. However, EMS is ten- and four-fold more mutagenic than MMS and iPMS, respectively, on an equitoxic basis.

It appears that, when comparisons are made at equimolar rather than equitoxic concentrations, the cytotoxic and mutagenic effects of the alkylsulphates and alkanesulphonates decrease with increasing size of the alkylating group. These results also reinforce the notion that cytotoxic and mutagenic effects of EMS are dissociable (Hsie *et al.* 1975a; O'Neill & Hsie, 1977). In addition, mutagenicity does not appear to associate with S_N1 reactivity, since iPMS has the highest S_N1 reactivity (Lawley, 1974) but is the least mutagenic of these five chemicals (Table 1).

Nitrosamidines and nitrosamides

The five *N*-nitroso compounds that have been studied have been shown to produce a concentration-dependent exponential loss of clonal growth. While ENU and BNU exerted cellular lethality with a shoulder region of 100 and 400 μM, respectively, MNNG, ENNG and MNU caused an exponential lethality without any appreciable shoulder (Couch &

Table 1. *Mutagenicity and cytotoxicity of alkylating chemicals**

Compound	Cytotoxicity†	Mutagenic activity‡ (mutants/10^6 cells) At 10% survival	Per μM mutagen
Alkylsulphates:			
Dimethylsulphate	90	90	1·0
Diethylsulphate	2800	780	0·3
Alkylsulphonates:			
Methyl methanesulphonate	100	140	1·5
Ethyl methanesulphonate	3700	1600	0·4
Isopropyl methanesulphonate	4500	440	0·1
Nitrosamidines:			
MNNG	0·3	200	590
ENNG	6	520	90
Nitrosamides:			
N-Nitroso-*N*-methylurea	90	240	3
N-Nitroso-*N*-ethylurea	1200	550	0·5
N-Nitroso-*N*-butylurea	2500	200	0·1

MNNG = *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine ENNG = *N*-Ethyl-*N'*-nitro-*N*-nitrosoguanidine

*Data from Couch, Forbes & Hsie (1978) and Couch & Hsie (1978).

†Cytoxicity is expressed as the concentration of chemical (μM) required to reduce cellular cloning efficiency to 10%.

‡Mutagenicity is expressed as (1) the number of mutants per 10^6 cells at 10% survival with no reference to chemical concentration, and (2) the slope of the linear portion of the mutation induction curve as the number of mutants/10^6 cells/μM of chemical tested.

Hsie, 1978). All of the agents, however, produced a linear increase of mutation induction throughout the entire survival curve (Couch & Hsie, 1978).

Of the two *N*-nitrosamidines that have been studied (Table 1) MNNG is more toxic than ENNG: concentrations of 0·3 and 6 mM produce 10% cell survival for MNNG and ENNG, respectively. When mutagenicity is compared on a molar basis, MNNG is six times more active than ENNG; however, ENNG is about three times more mutagenic than MNNG when the comparison is made at 10% survival because MNNG is 20 times more toxic than ENNG (Table 1).

The three *N*-nitrosamides have been found to be much less toxic than the two nitrosamidines that have been studied: 90, 1200 and 2500 μM are required to reduce survival by 90% for MNU, ENU and BNU, respectively. Apparently the bulkier the alkylating group, the less toxic it becomes. This trend also applies to mutagenicity: MNU is six and 35 times more mutagenic than ENU and BNU, respectively, when compared on an equimolar basis; however both MNU and BNU are only half as mutagenic as ENU at 10% survival (Table 1).

These results suggest that, of the five *N*-nitroso compounds studied, both cytotoxic and mutagenic effects decrease with an increase in the size of the alkylating group. *N*-Nitrosoguanidines are more effective mutagens than nitrosourea, since on a molar basis MNNG and ENNG are 200 times more potent than their corresponding nitrosoureas on a molar basis.

N-Methyl-*N'*-nitro-*N*-nitroguanidine, a non-nitrosated analogue of MNNG, has been shown not to be mutagenic even at concentrations 100,000 times higher than tested for MNNG (J. R. San Sebastian & A. W. Hsie, unpublished data, 1978). Nitrosation appears to be essential for nitrosamidines to be mutagenic.

Relationship between DNA alkylation and mutation at the *hgprt* locus by MNU and ENU

Once we had established the effect of the modification of the alkylating moiety on the mutagenicity of ten alkylating chemicals, we examined the correlation between the degree of DNA alkylation and the mutagenicity of selected chemicals. For this purpose, we chose MNU and ENU because of the existence of well-documented comparisons of mutagenicity in microbial systems (Lawley, 1974; Neale, 1976) and in the CHO/HGPRT assay (Couch & Hsie, 1978), of animal carcinogenicity (Pegg, 1977), and of chemical reactivity (Veleminský, Osterman-Golkar & Ehrenberg, 1970).

In cultures treated with MNU and ENU, we found that both alkylation and mutation induction by MNU and ENU increase linearly with increasing nitrosamide concentrations over the concentration range tested (Thielmann, Schröder, O'Neill, Brimer & Hsie, 1979). In this study, on an equimolar basis, MNU had 15 times the alkylating activity of ENU, but only three times the mutagenic activity. In terms of mutation induction per unit alkylation, ethylation of DNA by ENU appears to result in more than four times the number of mutagenic lesions than does methylation by MNU (Table 2). This may reflect either a higher miscoding frequency, such as O^6-guanine alkylation, or an effect of ethylation *per se* (Thielman *et al.* 1979).

Further studies will be directed toward determination of DNA adducts (alkylated nucleosides and phosphotriesters) and their relationship to mutation induction.

Platinum(II)chlorammines

Data that have been obtained for the six platinum compounds are shown in Table 3. Three platinum(II)

Table 2. *Mutation induction and DNA alkylation by MNU and ENU**

Compound	DNA alkylation†	Mutation induction‡	Ratio, mutation induction: DNA alkylation
MNU	57	2800	50
ENU	4	840	220
Ratio, MNU:ENU	15	3·3	

MNU = *N*-Nitroso-*N*-methylurea ENU = *N*-Nitroso-*N*-ethylurea

*Data from Thielmann, Schröder, O'Neill, Brimer & Hsie (1979).

†DNA alkylation is expressed as pmol of labelled radioactivity/μmol of cellular DNA/mM of unlabelled nitrosourea.

‡Based on the linear portion of the mutation induction curve; expressed as the number of mutants/10^6 cells selected/μM of nitrosourea.

compounds, *cis*-$Pt(NH_3)_2Cl_2$, $K[Pt(NH_3)Cl_3]$, and $[Pt(NH_3)_3Cl]Cl$, are mutagenic, the mutagenic potential of $K_2(PtCl_4)$ is marginal, and *trans*-$Pt(NH_3)_2Cl_2$ and $[(Pt(NH_3)_4]Cl_2$ are non-mutagenic. Based on the slope of the linear portion of the mutation induction curve, the relative mutagenicity of *cis*-$Pt(NH_3)_2Cl_2$: $K[Pt(NH_3)Cl_3]$: $K_2(PtCl_4)$: $[Pt(NH_3)_3Cl]Cl$: *trans*-$Pt(NH_3)_2Cl_2$: $[Pt(NH_3)_4]Cl_2$ is 100:9:0·4:0·3:0·04: 0·008; *trans*-$Pt(NH_3)_2Cl_2$ appears to lie on the lower limit of mutagenic response. Thus, *cis*-$Pt(NH_3)_2Cl_2$ is most mutagenic among these six platinum(II)chlorammines while its steric isomer, the *trans* form, is non-mutagenic; other structural congeners are less active or inactive in comparison with *cis*-$Pt(NH_3)_2Cl_2$. Analysis of the survival curve for treatment with *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ showed that the *cis* isomer is approximately 16 times more cytotoxic than the *trans* isomer, although the *cis* form is 2400 times more mutagenic than the *trans* form (Johnson, Hoeschele, Rahn, O'Neill & Hsie, 1980).

Binding of *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ to cellular DNA

Both isomeric forms of $Pt(NH_3)_2Cl_2$ were taken up by CHO cells and found to be bound covalently to cellular DNA. Determination of the slope of the number of *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ molecules bound per DNA nucleotide shows that the level of binding is three times higher per μM for the *cis* isomer than it is for the *trans* isomer (Table 3; Johnson *et al.* 1980).

A comparison of the mutagenicity and cytotoxicity per DNA lesion for *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ has shown that these two biological effects differ in their reactivity to DNA binding. The mutation induction by the *cis* isomer as a function of treatment concentration is 2400 times that of the *trans* isomer. However, the *cis* isomer binds only three times more to cellular DNA than does the *trans* isomer. The mutant yield per molecule bound to DNA is 800 times larger for the *cis* than for the *trans* isomer. Since 50% cellular lethality occurs at 1·3 and 35 μM for the *cis* and *trans* isomers, which correspond to 4×10^{-5} and 36×10^{-5} Pt molecules per DNA nucleotide, respectively, cytotoxicity per DNA lesion is a factor of nine greater for the *cis* than for the *trans* isomer. The *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ bind to cellular DNA to a similar extent (*cis*:*trans* = 3:1), yet the *cis* isomer is 2400 times more mutagenic and 16 times more cytotoxic than the *trans* isomer (Johnson *et al.* 1980). The subsequent expression and/or repair of these lesions

Table 3. *Mutagenicity, cytotoxicity and DNA binding of Pt(II)chlorammines**

Compounds	Mutagenic activity†		Cytotoxicity‡		
	mutants/10^6 cells/μM	max. no. of mutants/10^6 cells	log % survival/μM	LD_{50}	DNA binding§
cis-$Pt(NH_3)_2Cl_2$	31·5	140	−0·88	1·3	$2·8 \times 10^{-5}$
$K[Pt(NH_3)Cl_3]$	2·78	130	—	—	—
$[Pt(NH_3)_3Cl]Cl$	0·11	70	—	—	—
$K_2(PtCl_4)$	0·12	25	—	—	—
trans-$Pt(NH_3)_2Cl_2$	0·013	12	−0·005	35	$0·9 \times 10^{-5}$
$[Pt(NH_3)_4]Cl_2$	0·0025	17	—	—	—

*Data from Johnson, Hoeschele, Rahn, O'Neill & Hsie (1980).

†Expressed as (1) mutants/10^6 cells/μM based on the linear portion of the mutation induction curve, and (2) maximum number/10^6 cells derived from the highest observed number of mutants per 10^6 cells induced by a chemical among all concentrations tested. Spontaneous mutation frequency, 0–15 mutants/10^6 cells.

‡Expressed as (1) log % survival/μM based on the slope of the exponential portion of the survival (cloning efficiency) curve, and (2) LD_{50}, which is the concentration (μM) of a chemical required to reduce the cloning efficiency to 50%.

§The number of ^{195m}Pt-labelled molecules/DNA nucleotide/μM of platinum(II) compound.

appear to account for the difference in potency of the two biological end points in these two steric isomers.

Concluding remarks

Conditions necessary for quantifying mutation induction to TG resistance, which selects for mutants deficient in the HGPRT activity in CHO cells, have been defined. The quantitative nature of the CHO/HGPRT system permits studies of the effect of chemical structural modification on the mutagenicity and cytotoxicity of alkylsulphates, alkanesulphonates, *N*-nitrosamidines, *N*-nitrosamides, and platinum(II)-chlorammines. Analyses of DNA alkylation by MNU and ENU as well as DNA binding by *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ demonstrate that both the quantity and quality of DNA adducts affect mutagenicity and cytotoxicity. The CHO/HGPRT system appears to be useful for studying genetic aspects of toxicology and to relate molecular parameters necessary for mutagenic and cytotoxic activities.

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BIOASSAY FOR EVALUATING THE POTENCY OF AIRBORNE SENSORY IRRITANTS AND PREDICTING ACCEPTABLE LEVELS OF EXPOSURE IN MAN

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Summary—An animal bioassay has been used to evaluate the potency of a wide variety of airborne sensory irritants. Concentration-response relationships were obtained for each chemical tested and the potencies of these sensory irritants were compared. An attempt was made to predict from the data obtained in the animal bioassay what the response would be in humans. A good correlation was found between the Threshold Limit Values for these chemicals and the level that, on the basis of the potency of each chemical, would be predicted to be acceptable in humans.

Introduction

In 1966, Alarie proposed that the trigeminal nerve endings in the nasal mucosa in mice could be used to detect whether or not airborne chemicals would have sensory irritating properties in humans. A sensory irritant was defined as a substance that would evoke a painful burning sensation of the eye, nose or throat in man (Alarie, 1966 & 1973). However, instead of measuring neural activity from these nerve endings directly during exposure to airborne chemicals (Kulle & Cooper, 1975; Ulrich, Haddock & Alarie, 1972) the response measured was a decrease in respiratory rate. A decrease in respiratory rate due to stimulation of the trigeminal nerve endings by airborne irritants was first described by Kratschmer in 1870.

First, it was shown for a series of chemicals (Alarie, 1966 & 1973; Kane, Barrow & Alarie, 1979) that a perfect correlation existed between the characteristic decrease in respiratory rate in mice and subjective reports of sensory irritation of the eyes, nose and throat in humans. Secondly, it was shown that concentration-response relationships were easily obtained by plotting the percentage decrease in respiratory rate *v.* the logarithm of the exposure concentration for each airborne irritant (Alarie, 1966 & 1981; Kane *et al.* 1979; Kane, Dombroske & Alarie, 1980). Finally a major step in the development of this model was to select for investigation in mice a series of eleven airborne industrial contaminants, which were well recognized as sensory irritants in humans and for which the Threshold Limit Value (TLV) had been established *almost entirely* to prevent complaints of sensory irritation in industry (Kane *et al.* 1979). It was expected that some relationship could be found between the TLVs of these chemicals and their RD_{50} values (the concentrations necessary to evoke a 50% decrease in respiratory rate).

In two recent reports (Kane *et al.* 1979 & 1980), such relationships were presented and evaluated, and in a preliminary report (Alarie, 1981) data have been presented showing that a good correlation could be obtained between the TLV values and 0·03 RD_{50} values for each chemical in a series ranging from very potent to very weak sensory irritants. In this report, the results obtained for 26 chemicals are presented and the value of this animal bioassay is discussed.

Method

The method for measuring respiratory rate in mice during exposure to airborne chemicals has been described in detail (Alarie, 1966; Barrow, Alarie, Warrick & Stock, 1977). Briefly, the tidal volume of each mouse is recorded by body plethysmography and displayed on an oscillograph, so that the characteristic pause during expiration can be observed as an indication that the net decrease in respiratory rate is due to stimulation of the nasal trigeminal nerve endings. The average respiratory rate of four mice is displayed on a second oscillograph which permits continuous monitoring prior to, during and following exposure to airborne chemicals. In order to obtain concentration-response relationships, five to eight groups of animals are used for exposure to various concentrations of each airborne chemical. From these relationships, the RD_{50} is obtained and a comparison of potency is made on this basis.

Prediction of acceptable exposure concentration in industry

The chemicals previously tested are presented in Table 1 in order of descending potency. As a prediction for an industrial TLV, it was originally proposed that the TLV be set between 0·01 and 0·1 RD_{50} (Barrow *et al.* 1977). This suggestion was modified on the basis of the finding that, with repeated exposures at concentrations just above 0·1 RD_{50}, hypersusceptibility or cumulative effects could be detected for some chemicals such as acrolein, formaldehyde and toluene diisocyanate (Kane & Alarie, 1977; Sangha & Alarie, 1979). Therefore 0·1 RD_{50} was proposed as a ceiling level (Sangha & Alarie, 1979).

Table 1. *The RD_{50} values, 1980 TLV-TWA values* and TLV-TWA values predicted on the basis of 0·03 RD_{50} for 26 industrial chemicals*

Chemicals tested	RD_{50} (ppm)	1980 TLV-TWA (ppm)	0·03 RD_{50} (ppm)	log 1980 TLV-TWA (ppm)	log 0·03 RD_{50} (ppm)	Reference providing RD_{50} value
2,4-Toluene diisocyanate	0·20	0·005	0·006	−2·30	−2·22	Sangha & Alarie, 1979
Chlorobenzylidene malononitrile	0·52	0·05	0·016	−1·30	−1·80	Kane *et al.* 1979
Chloroacetophenone	0·96	0·05	0·029	−1·30	−1·54	Kane *et al.* 1979
Acrolein	1·68	0·1	0·05	−1·00	−1·30	Kane & Alarie, 1977
Formaldehyde	3·13	2	0·10	0·30	−1·00	Kane & Alarie, 1977
Benzoquinone	5·0	0·1	0·15	−1·00	−0·82	Y. Alarie, unpublished data 1979
Chloropicrin	7·98	0·1	0·24	−1·00	−0·62	Kane *et al.* 1979
Chlorine	9·34	1·0	0·28	0	−0·55	Barrow *et al.* 1977
Sulphur dioxide	117	2·0	3·5	0·30	0·54	Alarie, Wakisaka & Oka, 1973
Ammonia	303	25	9·1	1·40	0·96	Barrow, Alarie & Stock, 1978
Hydrogen chloride	309	5	9·3	0·70	0·97	Barrow *et al.* 1977
Dimethylamine	511	10	15	1·00	1·19	Steinhagen, Swenberg & Barrow, 1981
Ethyl acetate	614	400	18	2·60	1·26	Kane *et al.* 1980
Epichlorohydrin	687	2	20	0·30	1·31	Kane *et al.* 1979
Styrene	980	50	29	1·70	1·47	Y. Alarie, unpublished data 1979
Amyl acetate	1531	100	46	2·00	1·66	Kane *et al.* 1980
2-Butoxyethanol	2825	25	85	1·40	1·92	Kane *et al.* 1980
Isoamyl alcohol	4452	100	134	2·00	2·13	Kane *et al.* 1980
n-Butyl alcohol	4784	50	143	1·70	2·15	Kane *et al.* 1980
Acetaldehyde	4946	100	148	2·00	2·17	Kane *et al.* 1980
Methyl ethyl ketone	9000	200	270	2·30	2·43	Stone, Lawhorn, McKinney & McCracken, 1981
n-Propyl alcohol	12,704	200	381	2·30	2·58	Kane *et al.* 1980
Isopropyl alcohol	17,693	400	531	2·60	2·73	Kane *et al.* 1980
Ethanol	27,314	1000	819	3·00	2·91	Kane *et al.* 1980
Methanol	41,514	200	1245	2·30	3·10	Kane *et al.* 1980
Acetone	77,516	750	2325	2·88	3·37	Kane *et al.* 1980

TLV-TWA = Threshold Limit Value, time-weighted average

*When notice of intended change has been given (ACGIH, 1980) the intended future value is used. All values in the table are given in ppm for purposes of comparison, although for some chemicals the aerosol form instead of the gas phase was tested and the TLV-TWA would be given in mg/m^3.

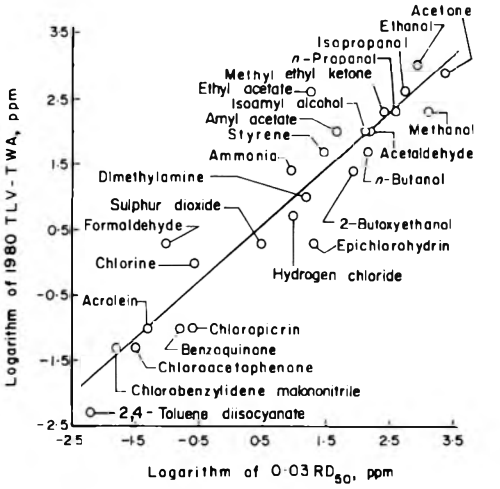

Fig. 1. Linear least squares regression analysis for 26 chemicals, plotting log of 0·03 RD_{50} as the proposed TLV-TWA *v.* log of TLV-TWA for each chemical. Data are taken from Table 1. Regression equation: $Y = 0{\cdot}126 + 0{\cdot}865\,X$. Standard deviation of Y about the regression line = 0·507, r^2 = 89·4%, r^2 adjusted for 24 degrees of freedom = 88·9%.

Since TLVs are established as a time-weighted average (TWA) concentration for a normal 8-hr workday, a suggestion was made that 0·03 RD_{50} be used as a TLV-TWA. This value, which on a logarithmic scale is the mid-point in the proposed range of 0·01–0·1 RD_{50}, was selected for practical reasons to prevent, as much as possible, excursions above 0·1 RD_{50} in industrial situations. If 0·03 RD_{50} is selected for use in predicting an acceptable TLV-TWA, a good relationship should exist between this value and the TLV-TWA for these chemicals. This should be especially apparent in instances in which the primary basis for establishing the TLV-TWA was to prevent sensory irritation. Such a relationship is presented in Fig. 1 using the data in Table 1 for each chemical tested.

The relationship was found to be good for this very wide variety of chemicals, which cover a potency range of five orders of magnitude. There are two compounds for which the animal model predicts a much lower TLV-TWA than the current one; these are formaldehyde and ethyl acetate. However, the documentation (American Conference of Governmental Industrial Hygienists—ACGIH, 1980) clearly recognizes that for these two chemicals, sensory irritation will occur *at* the TLV-TWA currently set. The relationship in Fig. 1 indicates one instance (epichlorohydrin) where the TLV-TWA is much lower than would be predicted by the model. The TLV-TWA was recently reduced for epichlorohydrin (ACGIH, 1980) on the basis of systemic toxicity.

Application of the model

This model can be useful in predicting a level of exposure likely to be acceptable in the industrial situation to prevent sensory irritation in humans. It would be foolish to think that a TLV can be established on the basis of this test alone. On the other hand, the results of this test indicate the maximum value likely to be acceptable for a TLV and can be used for engineering design and controls needed for the production of the chemical.

The results from this test can also be used for the design of longer-term inhalation studies. The 0·01 RD_{50} can be used as the low level and multiples of it for the higher levels. However, it is unlikely that levels approaching the RD_{50} will be of value for long-term inhalation studies. At this level, and above, the respiratory rate is depressed to a point where CO_2 retention is significant and important changes in acid-base balance occur. Since the RD_{50} concentration results in intolerable sensory irritation in humans (Kane *et al.* 1979), exposing animals to such levels in long-term chronic studies seems inappropriate. However, with several chemicals a fade in the sensory irritation response occurs in man as well as in mice. In these cases the decrease in respiratory rate occurring at the beginning of exposure diminishes with time. This occurrence was observed with sulphur dioxide, ammonia, formaldehyde and many of the solvents listed in Table 1. In such instances, it would be possible to use RD_{50} levels for long-term inhalation studies.

With other irritants, such as 2,4-toluene diisocyanate, benzoquinone and a series of isocyanates, the depression of respiratory rate was long lasting following a 3-hr exposure. With benzoquinone the respiratory-rate depression was long lasting following exposures as brief as 30 min. Repeated daily exposure to these chemicals at and above the 0·1 RD_{50} will result in cumulative effects, the net result being a depression in respiratory rate of 80% after 3–5 days of exposure (Sangha & Alarie, 1979; Sangha, Matijak & Alarie, 1981). This is an extremely stressful situation for the animals and would not be recommended for long-term studies. So far the model has been used primarily for single exposures. The few experiments conducted with repeated exposures, such as with the isocyanates noted above, indicate that much more can be learned about their toxicity with such protocols, and possible corrections could be made to allow the use of 0·03 RD_{50} to predict safe levels of exposure.

The model has also been used for evaluating the potency of a series of surfactants used in hair shampoos (Ciuchta & Dodd, 1978). The objective was to determine which of the detergents was the most or least potent sensory irritant by exposing mice to various airborne concentrations of each. Excellent concentration-response relationships were obtained and valid comparisons were made with eye-irritancy tests. Although the animal model measures sensory irritation caused by stimulation of nerve endings in the nasal mucosa, it should be remembered that the cornea is endowed with similar trigeminal nerve endings, although their anatomical location is slightly different (Kane & Alarie, 1977). A chemical that stimulates the nasal trigeminal nerve endings will also stimulate comparable nerve endings located in the cornea (Alarie, 1973). Thus the model can be used to evaluate irritation by cosmetic ingredients providing the ingredients can be converted to aerosols for the evaluation of their potential as sensory irritants. In fact, this animal model could be 'calibrated' in a way similar to that used to 'calibrate' the model for TLVs, by testing a series of ingredients used in cosmetics for which there are data on consumer complaints of eye irritation. However, the model would not replace the

eye-irritancy tests used to study the corrosive action of chemicals applied to the eye.

Concluding remarks

The trigeminal nerve endings are the 'common chemical sense' receptors (Alarie, 1973). They are stimulated by potentially noxious chemicals to warn man about the presence of such chemicals in his environment. In effect, they seem to 'measure' the reactivity of chemicals with nucleophiles (Alarie, 1973); the higher their reactivity the more potent the chemicals are as sensory irritants. It is therefore not so strange that if the concentration of a chemical is maintained at a level below which sensory irritation occurs, the likelihood that toxic effects will occur will be small. The fact that TLV levels established to prevent complaints of sensory irritation *seem* to be adequate to prevent the occurrence of other toxic effects from these chemicals gives support to this general idea. The main disadvantage of the model would be for chemicals that are of low reactivity but are metabolically activated. For these chemicals, as well as for ozone, phosgene and nitrogen dioxide, which are pulmonary irritants rather than sensory irritants, the model may predict a TLV-TWA that would be too high. This is under investigation. Another important point to consider is that the relationship presented in Fig. 1 was based on a single determination of the RD_{50} for each chemical. It would be more appropriate to have several determinations from different laboratories using chemicals of known purity.

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ACTION OF *N,N*-DIETHYLACETAMIDE ON HEPATIC MICROSOMAL DRUG-METABOLIZING ENZYMES

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Summary—The action of *N,N*-diethylacetamide on liver microsomal drug-metabolizing enzymes was studied *in vitro* using rat-liver homogenates, and *in vivo* in mice. *In vitro*, *N,N*-diethylacetamide had no effect on a series of mixed-function oxidases or on cytochrome *c* reductase but *in vivo*, these enzymes were inhibited, 45 min after a single, ip dose of 150 mg of the substance/kg body weight and there was also an increase in hexobarbital sleeping time. This discrepancy between the *in vitro* and the *in vivo* results may be due to oxidative *N*-dealkylation of diethylacetamide occurring *in vivo* leading to enzyme inhibition.

Introduction

In pharmacological and toxicological studies and in some other uses, water-insoluble compounds are often administered in water-miscible organic solvents such as dimethylsulphoxide, glycerol, propylene glycol, polyethylene glycol, and *N,N*-diethylacetamide. It has been shown that these vehicle compounds are not as biologically inert as they were originally thought to be (Budden, Kühl & Bahlsen, 1979; Budden, Kühl & Buschmann, 1978a,b,c; Gergely, 1970). Budden *et al.* (1978a,b,c) showed that amongst other effects, they can prolong hexobarbital sleeping time. Thus these substances can interfere with the biotransformation and the pharmacokinetics of compounds to be tested in pharmacological or toxicological studies.

We studied the effect of *N,N*-diethylacetamide on oxidative drug metabolism *in vivo* and *in vitro* because this solvent has structural similarities to the aliphatic amide, 2-hydroxy-2-ethylbutyryl *N,N*-diethylamide (HOE 17,879), a compound which has been shown to prolong drug action times (Beyhl & Lindner, 1976; Lindner, 1960) and to inhibit hepatic microsomal drug-metabolizing mixed-function oxidases, both *in vivo* (Beyhl & Lindner, 1973 & 1976) and *in vitro* (Beyhl, 1980a), but to have no effect on hepatic microsomal NADPH-dependent cytochrome *c* reductase *in vivo* (Beyhl & Lindner, 1976) or *in vitro* (Beyhl, 1980a).

Experimental

Materials. The following chemicals were used: saccharose, inorganic salts, and buffer substances obtained from Merck (Darmstadt) and Riedel-DeHaën (Seelze), biochemicals obtained from Boehringer (Mannheim), *N,N*-diethylacetamide and coumarin obtained from Merck, aminopyrine obtained from Hoechst (Frankfurt), sodium hexobarbital obtained from Bayer (Leverkusen), and 4-methylumbelliferone obtained from EGA-Chemie (Steinheim). 4-Methoxybiphenyl and 7-ethoxycoumarin were the gifts of Dr Sinharay (Hoechst).

In vivo *study*

Treatment of animals. Male mice of the NMRI strain obtained from our breeding station, were divided at random into two groups of 16 animals. The mice of one group were injected ip with an aqueous solution containing 150 mg *N,N*-diethylacetamide/kg body weight and those of the other (control) group were injected with the same amount of water. After 45 min six animals from each group were injected iv with 100 mg sodium hexobarbital/kg body weight for the measurement of hexobarbital sleeping time according to Lindner (1960). The remaining animals were killed by a blow on their heads and bleeding; the livers were removed, weighed, and frozen immediately by immersion into liquid nitrogen. The livers were stored at −20°C until further handling. Activities of drug-metabolizing enzymes were not deteriorated by this procedure (F. E. Beyhl, unpublished data 1980).

Biochemical measurements. After thawing, the livers were homogenized in ice-cold isotonic saccharose solution with a glass/polytetrafluoroethylene homogenizer of the Potter-Elvehjem (1936) system driven by compressed-air motor (Pressluft-Götz, Mannheim). From these crude homogenates, microsomes were prepared by the calcium chloride precipitation method as cited previously (Beyhl & Mayer, 1980). The activities of certain enzymes in these microsomes were assayed as described below. Methoxybiphenyl *O*-demethylase was assayed according to Beyhl (1980b; cf. Bridges, Creaven & Williams, 1965; Creaven, Davies & Williams, 1967; Davies & Creaven, 1964). Aminopyrine *N*-demethylase was determined following Leber, Degkwitz & Staudinger (1969; cf. Volz & Kellner, 1980). Coumarin 7-hydroxylase was assayed by our own method (F. E. Beyhl, unpublished data, 1981; cf. Creaven, Parke & Williams, 1965; Fink & von Kerékjártó, 1966; von Kerékjártó, 1966; von Kerékjártó, Kratz & Staudinger, 1964) with 4-methylumbelliferone as the fluorescence standard, and microsomal NADPH-dependent cytochrome *c* reductase was measured according to Cleveland & Smuckler (1965). All the resulting values were tested for statistical significance using Student's *t* test (Ther, 1965).

In vitro *studies*

For the *in vitro* studies, the livers of untreated Wistar

rats, of either sex, obtained from our breeding station were homogenized either in isotonic saccharose solution for the preparation of calcium chloride-precipitated microsomes as described above or in isotonic potassium chloride solution for the preparation of 13,000 g supernatants (Beyhl & Mayer, 1980). In the 13,000 g supernatants, the activities of aminopyrine *N*-demethylase and cytochrome *c* reductase were determined as outlined above; in previous studies it had been shown that these microsomal enzymes could be measured without disturbance of the cytosolic components present in these supernatants (F. E. Beyhl, unpublished data, 1980). In the microsomal preparation, the activities of methoxybiphenyl *O*-demethylase and coumarin 7-hydroxylase were measured by the methods cited above, and that of 7-ethoxycoumarin *O*-deethylase was determined by our own method (F. E. Beyhl, unpublished data, 1981; cf. Aitio, 1978; Ullrich & Weber, 1971). All the enzyme activities were assayed in the absence and in the presence of the *N,N*-diethylacetamide at concentrations of 0·1, 1·0 and 10 mM, in the assay medium (cf. Beyhl, 1980a). Again, the resulting values were tested for statistical significance using Student's *t* test.

Results

The results are listed in Tables 1 and 2. All the enzyme activities measured in the *in vivo* experiment were significantly decreased and hexobarbital sleeping time was doubled. In the *in vitro* experiment *N,N*-diethylacetamide did not have a significant effect on the activity of any of the enzymes.

Discussion

The lack of enzyme inhibition observed *in vitro* shows that *N,N*-diethylacetamide does not inhibit either mixed-function oxidases or cytochrome *c* reductase. Other microsomal enzyme inhibitors either inhibit the mixed-function oxidases, as a result of interaction with cytochrome *P*-450, the terminal oxidase of the microsomal redox chain (Beyhl, 1980a,c; Cooper, Axelrod & Brodie, 1954) or inhibit both the mixed-function oxidases and cytochrome c reductase (Beyhl, 1981). Thus *N,N*-diethylacetamide would not be expected to affect drug-metabolizing enzymes or hexobarbital sleeping time *in vivo*. However our results show that *N,N*-diethylacetamide reduces both mixed-function oxidase and cytochrome *c* reductase activities *in vivo* and, as a consequence of the thus impaired drug biotransformation, prolongs hexobarbital sleeping time. The increased hexobarbital sleeping time was also observed by Budden *et al.* (1978a). In view of the *in vitro* results this effect on drug-metabolizing enzymes cannot be explained as resulting from enzyme inhibition by *N,N*-diethylacetamide itself. We would like to suggest that *in vivo*, *N,N*-diethylacetamide is *N*-dealkylated to form a reactive metabolite which blocks both drug metabolism by mixed-function oxidases and microsomal electron transport by cytochrome *c* reductase. A similar mechanism for inhibition of drug-metabolizing enzymes is being discussed for diethylamino-ethyl 2,2-diphenylvalerate hydrochloride (SKF 525-A; Proadifen), a well-known inhibitor of mixed-function oxidases (Barber & Wilson, 1980; Schenkman, Wilson & Cinti, 1972). Studies on this metabolic activation of *N,N*-diethylacetamide are in progress.

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Table 1. *Action of* N,N-*diethylacetamide on hepatic parameters in mice* in vivo

Group	Hexobarbital sleeping time (min)	Aminopyrine *N*-demethylase (U/g liver)	Methoxybiphenyl *O*-demethylase (U/g liver)	Coumarin 7-hydroxylase (mU/g liver)	Cytochrome *c* reductase (U/g liver)
Control	50 ± 11	0·452 ± 0·031	0·179 ± 0·01	5·06 ± 0·93	31·5 ± 3·5
Treated	101 ± 17 (202%)	0·315 ± 0·047 (69·7%)	0·127 ± 0·02 (70·9%)	2·72 ± 1·07 (53·8%)	24·5 ± 3·1 (77·8%)

Values are means ± 1 SD for groups of ten animals (except hexobarbital sleeping time, six animals). Values in parentheses are percentages of the corresponding control value. All values for the treated group differ significantly (Student's *t* test) from those of the corresponding control group ($P < 0.0005$).

Table 2. *Effect of* N,N-*diethylacetamide on rat-liver enzyme activities* in vitro

N,N-Diethylacetamide concn (mM)	Enzyme activities (% of control)				
	Aminopyrine *N*-demethylase	Methoxybiphenyl *O*-demethylase	Ethoxycoumarin *O*-deethylase	Coumarin 7-hydroxylase	Cytochrome *c* reductase
0 (control)	100 ± 3·9	100 ± 1·5	100 ± 8·5	100 ± 3·4	100 ± 2·6
0·1	105·2 ± 11·2	99·2 ± 1·9	100·7 ± 9·2	96·8 ± 3·1	99·8 ± 2·8
1·0	102·1 ± 22·8	103·9 ± 2·7	100·4 ± 6·5	99·2 ± 7·9	111·8 ± 2·7
10	86·4 ± 13·2	106·2 ± 2·4	96·5 ± 5·3	82·8 ± 7·4	111·5 ± 5·0

Values are means ± 1 SD. None of the values differs significantly (Student's *t* test) from that of the corresponding control group.

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THE IMPACT OF AIR-LEAD ON BLOOD-LEAD IN MAN—A CRITIQUE OF THE RECENT LITERATURE*

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Summary—The nature of the relationship between air-lead and blood-lead in man was evaluated using all the available information from published studies in which subjects were in an approximately steady state with regard to lead exposure, and blood-lead could be related to air-lead measurements made in the individual's breathing zone. The observed blood-lead–air-lead relationship was compared with the relationships predicted by applying the two kinetic models which have been proposed to describe lead disposition in man. The models were found to be inconsistent with observed lead disposition kinetics. Each model generates a linear blood-lead–air-lead relationship with predicted blood-lead levels well above the actual levels except within the very lowest blood-lead range ($< 5\ \mu g/m^3$). The observed blood-lead–air-lead relationship is curvilinear with slope decreasing as blood- and air-lead increase. The implications of this curvilinearity with regard to the dependence of α, the increment in blood-lead associated with a given increment in air-lead, both on the magnitude of the air-lead increment and on the baseline blood-lead value are illustrated and discussed.

Introduction

A document published in 1972 (NAS-NRC, 1972) was intended to review and evaluate all the literature bearing on the question of the significance of airborne lead for human health and welfare. The committee that prepared this document was unable to arrive at a firm conclusion concerning the contribution of lead in air to the total body burden. It identified two general exposure routes, the inhalation of lead aerosols and the hand-to-mouth transfer of lead-bearing street dust, a potential problem among young children.

More recently, a World Health Organization task group (WHO, 1977) and the US Environmental Protection Agency (EPA, 1977) have been confronted with the same issues. All three of these study groups considered the possibility of estimating the importance of air-lead by calculating directly transfer from air (or street dust) into the body. This approach was found by the NAS-NRC study group to be impractical because of the uncertainties surrounding airway deposition and clearance rates for the lead aerosols of interest. The importance of hand-to-mouth transfer of fallout lead in street dust was even more difficult to assess because, in addition to uncertainties regarding the gastro-intestinal absorption of lead in children, there was absolutely no information concerning how much street dust a child might swallow. This particular problem, therefore, was simply cited as being one which required attention.

With regard to the impact of the inhalation of lead aerosols, the NAS-NRC committee concluded from the sparse epidemiological data available at the time that there was no perceptible impact of the concentration of lead in air (PbA) on the concentration of lead in blood (PbB) below a PbA level of 2–3 $\mu g/m^3$. The committee recommended that more precise studies be conducted concerning the relationship between ambient-air-lead concentration and blood-lead concentration, perhaps by use of personal monitors. This specific recommendation was implemented in one study (Azar, Snee & Habibi, 1973a).

Between the time of publication of the NAS-NRC document and the preparation of the WHO document, which actually took place in 1975, some new information became available concerning the disposition of lead, both inhaled and swallowed. Still more information was available to the US EPA in the preparation of its 1977 document. Yet, in both documents summary judgement as to the impact of inhaled lead on the body burden relied mainly on epidemiologic information concerning the relationship between PbB and PbA. The impact of lead in street dust on the body burden of lead in children was judged in both documents still to be surrounded by great uncertainty.

The present critique will take a fresh look at the impact of air-lead on blood-lead in man, considering all the useful information currently available. The problem still is basically divisible into two, the inhalation of lead aerosols and the hand-to-mouth transfer of fallout lead in the environment. Consideration will be given only to the first problem.

There are two approaches to estimating the impact of inhaled lead aerosols on the body burden of lead. The first is to use measured rates of uptake (or loss) of lead from an experimental study together with a disposition model to project either total body burden or the amount of lead in any of the presumed kinetic compartments at any time. This approach must take

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into consideration uptake from all sources by all absorption routes since the concern is with the relative importance of inhaled lead to the total body burden.

Two different disposition models have been used to describe lead kinetics. Rabinowitz, Wetherill & Kopple (1976) applied a three-compartment model to human exposure data from their studies of the kinetics of stable labelled lead. Bernard (1977) proposed a five-compartment model to describe human lead kinetics. Both models are shown schematically in Fig. 1.

The second approach to estimating the impact of inhaled lead aerosols on the body burden of lead is purely empirical: the observed relationship between PbA and PbB is evaluated mathematically. For this approach to be valid certain criteria must be satisfied. First, steady state or near steady state conditions must prevail, wherein the amount of lead inhaled per unit time must have been reasonably constant over a period of time long enough for virtual equilibration to occur between PbA and PbB. The input of lead from other sources must also be approximately constant, since it is not negligible relative to the input of lead from air.

In this paper we will evaluate the nature of the PbB–PbA relationship within the range of normal ambient-air lead concentrations as observed by Azar *et al.* (1973a). PbB values predicted by this relationship both within and beyond the range of PbA levels on which it is based will be compared with individual data points from experimental studies and with those predicted by application of the Rabinowitz and Bernard models. All the data used apply only to adults.

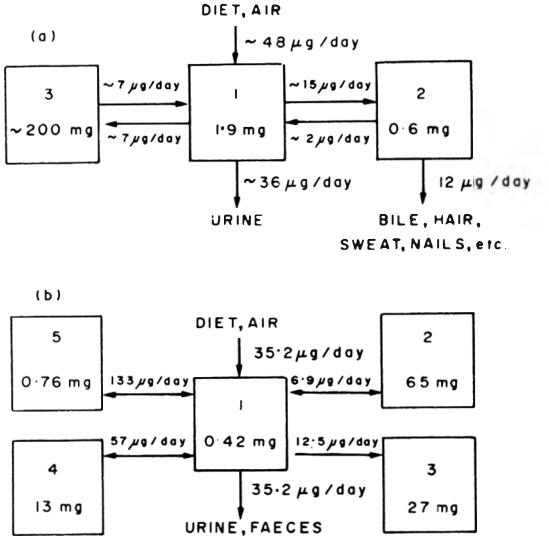

Fig. 1. Models proposed to describe lead disposition kinetics by (a) Rabinowitz *et al.* (1976) and (b) Bernard (1977). The amounts of lead in each compartment, exchanging daily between compartments, and excreted daily are shown for steady state conditions of continuous exposure to food, water, and air lead. The amounts of lead shown entering the central compartment in each case are the amounts that are assumed to be absorbed daily into the systemic circulation from all sources.

Observations: graded exposure levels

Azar *et al.* (1973a) monitored the PbA exposure of 150 adults from five locations in the United States, continuously and individually for 2–4 wk. The subjects were in a steady state condition with regard to exposure; the average number of years spent on the current job varied from 16–19·1 at the five different locations. Air-lead exposure, calculated as a time-weighted average, was monitored using personal air samplers and ranged up to about 4 μg total PbA/m^3 in the group with the lowest exposures and up to about 9 μg total PbA/m^3 in the group with the highest exposures. The concentration of respirable lead was somewhat less.

Statistical analysis of these data could be performed using regression models of varying degrees of complexity. For this calculation it was decided to use the simplest model available; that is, a straight-line fit with PbB depending upon PbA, and also to include a component representing differences among the five geographic locations. Further, the values of PbA and PbB could first be transformed, e.g. by taking their logarithms, or they could remain untransformed. However, any particular choices in this respect might well be criticized as being somewhat arbitrary. In this analysis, therefore, it was decided to use an empirical approach to the problem of transforming PbA and PbB (Box & Cox, 1964) to obtain the optimal transformations. This requires that a variable of interest, Y, be transformed by the expression $(\gamma^{\lambda} - 1)/\lambda$ where λ is chosen so that the assumptions of normality and homogeneity of variance in regression analysis are best satisfied.

In order to avoid unrealistic predicted values for PbB when PbA = 0, the component C_i, where $i = 1, \ldots, 5$, representing each of the five geographic locations, was incorporated into the model as follows:

$$PbB = A + B(PbA + C_i),$$

C_i represents the non-air lead contribution to PbB, expressed in terms of equivalent PbA.

The Azar *et al.* data were analysed by applying the above transformation both to PbB and PbA, and the optimal parameters in the model were found to be

$$(PbB)^{-1\cdot019} = -0\cdot09786 + 0\cdot17904(PbA + C_i)^{-0\cdot104}$$

where $C_1 = 6\cdot55627$, $C_2 = 1\cdot61865$, $C_3 = 0\cdot38578$, $C_4 = 5\cdot98683$, and $C_5 = 2\cdot76079$ for the five locations, with $R^2 = 0\cdot49$, the proportion of variation explained by the model. The average relationship between PbB and PbA was found by substituting the average C_i value, 3·46186, and solving for PbB:

$$PbB = [-0\cdot09786 + 0\cdot17904(PbA + 3\cdot46186)^{-0\cdot104}]^{-1/1\cdot019} \quad (1)$$

This result can be interpreted as the reciprocal transform for PbB and almost the logarithmic transform for PbA. The PbB–PbA relationship, shown in Fig. 2, is curvilinear with slope decreasing as PbA and PbB increase.

Observations: discrete exposure levels

The criteria for including data points in this analysis were: first, that the individuals studied were in an

approximately steady state of lead exposure; and second, that PbB could be related to PbA measurements made in the individual's breathing zone. Two studies were considered to meet these criteria adequately. Both involved the exposure of human volunteers to known concentrations of artificially generated lead sesquioxide.

Kehoe (summarized by Gross, 1979) exposed individuals singly, and often the same individuals sequentially, at many different air-lead concentrations for periods of time ranging from 98 to 772 days. Griffin, Coulston, Wills, Russell & Knelson (1975) exposed groups of volunteers for 14–19 wk at only two air-lead concentrations. Hence, standard deviations were calculated for the Griffin *et al.* data. It is assumed that in both of the above studies the PbAs monitored by the investigators were the concentrations actually in the breathing zone of the subjects. Hand to mouth transfer of settled lead-bearing dust probably was minimal. Kehoe subjects dusted the chambers frequently (Kehoe subject JOS, personal communication 1980). A similar housekeeping program prevailed in the Griffin study (T. B. Griffin, personal communication, 1980).

In order to minimize the effect on these data of non-air sources of lead, all the experimental data points were normalized to the same control value. For this purpose, the control period PbA range of 0·1–0·2 $\mu g/m^3$ measured in the Griffin *et al.* study was used. No control period PbA measurements had been reported for the Kehoe study. From the line of best fit to the Azar *et al.* data, the PbB values corresponding to PbA values of 0·1–0·2 $\mu g/m^3$ were found to be 16·1–16·2 $\mu g/dl$. Since the Kehoe PbB data were given only to two significant figures, these values were rounded off and a control period PbB of 16 $\mu g/dl$ was used to normalize experimental data. The adjustment was made by subtracting from individual or mean PbB values, as appropriate, the difference between measured control period PbB and 16 $\mu g/dl$.

These data are summarized in Table 1 and shown in Fig. 2.

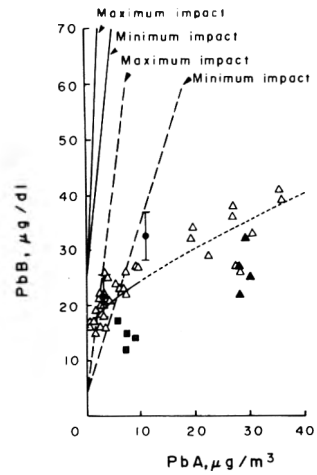

Fig. 2. Predicted and observed PbB level as a function of PbA. Data are taken from Table 1. The Kehoe data points (△▲■) represent 12 individuals, some studied more than once; data points from individuals exposed to large particle aerosols (▲); data points from one individual, DH, whose measured PbB from non-air lead sources was exceptionally high (■). All data points are adjusted to a PbB from non-air lead sources of 16 $\mu g/dl$. Standard deviations are shown for Griffin *et al.* points (●). The line of best fit to the Azar *et al.* data is shown dashed beyond the range of actual measurement. The Rabinowitz (——) and Bernard (– –) predicted lines (after 5 yr) are taken from Table 2; maximum and minimum impact conditions are discussed in the text.

Prediction: linear kinetic models

The differential form of the Rabinowitz model (Fig. 1)* is

$$dM_1/dt = (0{\cdot}002/0{\cdot}6)M_2 + (0{\cdot}007/200)M_3 - (0{\cdot}015 + 0{\cdot}007 + 0{\cdot}036/1{\cdot}9)M_1$$
$$dM_2/dt = (0{\cdot}015/1{\cdot}9)M_1 - (0{\cdot}002 + 0{\cdot}012/0{\cdot}6)M_2$$
$$dM_3/dt = (0{\cdot}007/1{\cdot}9)M_1 - (0{\cdot}007/200)M_3$$

where dM_1/dt, dM_2/dt and dM_3/dt represent the rates of change in the mass M of lead in compartments 1, 2 and 3 respectively as determined by rates of transfer into and out of the three compartments. In these equations time t is in days and the coefficients of the various Ms have the dimension [day^{-1}].

*Both models are given here for disposition only; that is, it is assumed that intake has ceased. If exposure continues throughout the disposition phase, functions describing the rates of input must be incorporated into the equations for dM_1/dt.

The differential form of the Bernard model* is

$$dM_1/dt = (0{\cdot}133/0{\cdot}76)M_5 + (0{\cdot}057/13)M_4 + (0{\cdot}0125/27)M_3 + (0{\cdot}00686/65)M_2 + (0{\cdot}2442/0{\cdot}419)M_1$$
$$dM_2/dt = (0{\cdot}00686/0{\cdot}419)M_1 - (0{\cdot}00686/65)M_2$$
$$dM_3/dt = (0{\cdot}0125/0{\cdot}419)M_1 - (0{\cdot}0125/27)M_3$$
$$dM_4/dt = (0{\cdot}057/0{\cdot}419)M_1 - (0{\cdot}057/13)M_4$$
$$dM_5/dt = (0{\cdot}133/0{\cdot}419)M_1 - (0{\cdot}133/0{\cdot}76)M_5$$

where time t is in days and five equations are necessary to describe the net rates of change in the five model compartments.

The compartments depicted do not have discrete anatomical dimensions. Thus, compartment 1 in both models includes but is not necessarily limited to blood. Therefore, it is not surprising that the amounts of lead associated with specific compartments are different in the two models.

The PbB levels predicted by the Rabinowitz and the Bernard models after exposure for 5 yr (260 wk) and 19 yr (1000 wk) were computer simulated in the following way. The amount of lead absorbed each day into the systemic circulation was entered into the computer program as a single unit. This daily increment was allowed to be distributed and excreted in accordance with the magnitudes of the model rate constants (that is, in accordance with the equations given). The initial condition was the complete absence of lead from all body compartments. Table 2 gives the predicted PbB levels at the end of the stated exposure periods as functions of the total amount of lead absorbed each day from all sources.

Table 1. *Individual and mean steady state PbB levels in persons exposed to lead sesquioxide aerosols and values derived from the line of best fit to the data of Azar* et al. *together with the associated values of* α

Subject or no. of subjects	Control period* PbB (μg/dl)	Experimental period PbA† (μg/m³)	Experimental period PbB (μg/dl)	Experimental period PbB (μg/dl) adjusted to baseline of 16 μg/dl	α‡	References
MB	24	29·4§	40	32	0·55	Kehoe (Gross, 1979)
MOB	26	22·4	39	29	0·58	
	26	28·4§	32	22	0·21	
PB	23	28·4	33	26	0·35	
SB	21	27·4	32	27	0·40	
FC	22	30·6	39	33	0·56	
	22	30·1§	31	25	0·30	
LD	18	9·3	29	27	1·21	
	18	19·7	36	34	0·92	
	18	27·1	40	38	0·82	
	18	35·9	41	39	0·64	
DH	30	5·6	31	17	0·18	
	30	7·3	26	12	−0·56	
	30	7·6	29	15	−0·14	
	30	8·8	28	14	−0·23	
NK	20	0·6	20	16	0	
	20	1·2	21	17	1·00	
	20	1·9	23	19	1·76	
	20	2·4	26	22	2·73	
	20	3·3	26	22	1·94	
	20	3·6	30	26	2·94	
	20	4·0	29	25	2·37	
HR	21	2·4	25	20	1·82	
	21	3·7	21	16	0	
	21	7·5	27	22	0·82	
JOS	21	9·4	32	27	1·20	
	21	19·3	37	32	0·84	
	21	27·1	41	36	0·74	
	21	35·7	46	41	0·70	
JUS	21	28·1§	32	27	0·39	
SS	19	0·6	20	17	2·5	
	19	1·3	20	17	0·91	
	19	1·8	18	15	−0·62	
	19	2·4	19	16	0	
	19	2·7	23	20	1·60	
	19	3·4	24	21	1·56	
	19	4·3	24	21	1·22	
	19	2·4	24	21	2·27	
	19	3·2	21	18	0·67	
	19	5·4	27	24	1·54	
	19	6·2	26	23	1·17	
	19	7·0	26	23	1·03	
	19	7·2	29	26	1·43	
8	20·3 ± 3·2	10·9	36·8 ± 4·3	32·5 ± 4·3	1·54	Griffin *et al.* (1975)
12	20·3 ± 4·2	3·2	26·0 ± 3·8	21·7 ± 3·8	1·90	
		1·0		17·108	1·135	Line of best fit to data of Azar *et al.* (1973a)
		2·0		18·133	1·074	
		5·0		20·741	0·946	
		10·0		24·285	0·825	
		20·0		30·151	0·704	
		30·0		35·378	0·644	
		40·0		40·386	0·608	

* The PbA during control periods is not known for the Kehoe studies but was 0·1–0·2 for the Griffin *et al.* (1975) studies. For calculation of α (see footnote ‡), baseline PbA was assigned the value 0·2 μg/m³ for the Kehoe studies.

†All PbA levels are expressed as if exposure had been continuous. For example, if a subject were exposed for 8 hr/day, 5 days/week, to a chamber air-lead concentration of 150 μg/m³, the value in the table would be [(8 hr/day)(5 day/wk)/(168 hr/wk)] 150 μg/m³ = 35·7 μg/m³.

‡α is the ratio ΔPbB:ΔPbA, where ΔPbB is the magnitude of the increment in PbB resulting from an increment in PbA of ΔPbA. The values of ΔPbB are calculated as the PbB values in column 5 minus 16 μg/dl, the baseline value. The values of ΔPbA are calculated as the PbA values in column 3 minus 0·2 μg/m³. Calculation of α from the line of best fit to the Azar *et al.* data starts from the baseline assumptions that PbA = 0·2 μg/m³ and PbB = 16·2 μg/dl. The value of α depends on the choice of baseline; see text for a discussion of this point.

§Large-particle aerosols.

These values are means ± 1SD.

Table 2. *The impact of PbA on PbB as predicted by the Bernard and the Rabinowitz models under different conditions*

Total Pb absorbed daily from all sources (μg)	PbB level predicted by Bernard model (μg/dl)		PbB level predicted by Rabinowitz model (μg/dl)		PbA corresponding to daily retention; 'minimum impact' conditions* (μg/m³)	PbA corresponding to daily retention; 'maximum impact' conditions† (μg/m³)
	After 5 yr (260 wk)	After 19 yr (1000 wk)	After 5 yr (260 wk)	After 19 yr (1000 wk)		
35·2	13·5	16·5	54·3	55·4	3·05	1·28
70·4	30·5	34·2	110·0	111·9	8·63	3·64
105·6	48·4	52·4	166·8	169·4	14·22	5·99
140·8	66·7	70·9	224·5	227·7	19·81	8·35
176·0	85·2	89·6	283·0	286·7	25·40	10·70
211·2	103·9	108·3	342·1	346·1	30·98	13·06

*'Minimum impact' conditions are chosen to minimize the predicted impact of PbA on PbB. They are: total volume of air breathed daily, 18 m³; 35% of inhaled Pb deposited in lung and absorbed. Lead absorbed from diet (food plus water) is assumed to be constant at 16 μg (0·08 × 200 mg)/day.

†'Maximum impact' conditions are chosen to maximize the predicted impact of PbA on PbB. They are: total volume of air breathed daily, 23 m³; 65% of inhaled Pb deposited in lung and absorbed. Lead absorbed from diet is assumed to be 16 μg/day.

The total lead absorbed daily was considered to be the sum of the amounts absorbed from the diet and from the air. It was assumed that total dietary (food plus water) lead was constant at 200 μg/day (Rabinowitz *et al.* 1976) and that 8% of this dietary lead, or 16 μg, was absorbed. The remainder of the amount absorbed was attributed to air-lead.

To convert the amount of lead absorbed from air to a concentration of lead in air, certain assumptions must be made about the volume of air breathed daily and about the fraction of inhaled lead absorbed from the lung. Chamberlain, Heard, Little, Newton, Wells & Wiffen (1978) have shown that the fraction absorbed is dependent on particle size. At the same time they investigated the particle size distribution of lead in ambient air. They found that in general the diffusional mean equivalent diameter (DMED) of mature aerosols such as those typical of rural areas and of urban areas away from the influence of major traffic arteries was about 0·06 μm while the mass median equivalent diameter (MMED) was larger, about 0·2–0·3 μm. The DMED of freshly generated lead aerosols measured near major highways was smaller, 0·03 μm, and the MMED was much smaller, 0·04 μm.

In our simulations two sets of assumptions were evaluated separately: one consistent with maximum impact of PbA on PbB, and one consistent with minimum impact of PbA on PbB. For maximum impact it was assumed that the individual is "reference man" and breathes 23 m³ air/day (ICRP, 1975) and that 65% of the lead inhaled is deposited in the lung and absorbed into the systemic circulation. This absorption level approximates the midpoint of the range reported by Chamberlain *et al.* (1978) for lead aerosols with very small DMED, 0·02–0·94 μm, at breathing cycles of 4–8 sec. For minimum impact it was assumed that reference man breathes 18 m³ air/day and that 35% of the lead inhaled is absorbed. This absorption magnitude approximates the midpoint of the range reported by Chamberlain *et al.* for lead aerosols having a larger DMED, about 0·09 μm at breathing cycles of 4–8 sec. The PbB levels predicted by the Rabinowitz and Bernard models after exposure for 5 yr are shown in Fig. 2.

Results and discussion

Figure 2 allows direct comparison of the PbB–PbA relationships predicted by the Rabinowitz and the Bernard models, calculated from the data of Azar *et al.* and those observed in experimental studies.

It should be noted that at steady state the Bernard model will generate a PbB level of 19·0 μg/dl for a total daily absorption of lead from all sources of 35·2 μg, corresponding for the conditions of this simulation to a PbA of between 1 and 3 μg/m³ depending on whether maximum impact or minimum impact conditions are considered. However, because of the very slow return of lead from two of its peripheral compartments (compartments 2 and 3, Fig. 1) the Bernard model has not yet achieved a steady state even after a 19-yr simulated exposure (Table 2).

Figure 2 shows that the line of best fit to the Azar *et al.* data provides a good fit to the data points from experimental studies while simulation based on the kinetic models of either Rabinowitz or Bernard predicts entirely inappropriate PbB values. One of the reasons for the poor quality of fit is that both kinetic models are linear while the observed relationship between PbB and PbA is curvilinear.

Both the Rabinowitz and the Bernard models are linear mammillary models consisting of a central compartment into which the foreign compound enters and from which it is eliminated by excretion or metabolism. Exchange takes place continuously between this central compartment and one or more peripheral compartments into which the compound is distributed. These exchanges as well as the elimination processes are first order (kinetically linear); that is, the unidirectional transfer rate is directly proportional to the amount available for transfer. Compartments as defined kinetically are not necessarily congruent with specific tissues or organ systems; however, the blood is usually considered to be part of the central compartment. In general, linear mammillary

models differ from one another only in the number of peripheral compartments assigned to each and in the values of the transfer rate constants.

The utility of linear kinetic models in describing the behaviour of foreign compounds, particularly of drugs, is firmly established. Linear models are founded on reasonable assumptions about the kinetic nature of transfer across biological membranes, assumptions which have been verified experimentally in many instances. Furthermore, they are the simplest biologically reasonable models. Thus a linear mammillary model is generally the first choice in modelling the kinetics of a foreign compound unless specific information about observed kinetic behaviour dictates otherwise.

The mathematical expression for the time-dependence of the amount in the central compartment of a linear mammillary model after acute administration (the integrated form of the differential equations given) leads directly to the identification of certain attributes shared by all linear mammillary models. One of these is a direct proportionality between the amount of material in each compartment and dose or dose rate at steady state. The theoretical proportionality constant is a function of the excretion rate constant as well as of the dose or dose rate. One implication of representing the kinetic behaviour of a foreign compound by a linear mammillary model is that tissue concentration should be proportional to exposure at steady state. However, it is clear from Fig. 2 that the PbB–PbA relationship is not linear at steady state. The linear models are not accurate representations of lead disposition kinetics.

A measure in common use to describe the PbB–PbA relationship is α or ΔPbB–ΔPbA, the increment in PbB associated with a given increment in PbA. As a result of the curvilinearity of the PbB–PbA relationship, α is not a constant. To begin with, α is dependent on the magnitude of the PbA increment used in its calculation. This dependence is illustrated in Fig. 3, in which α values for individual or mean data points from the Kehoe and Griffin *et al.* studies can be compared with α calculated from the line of best fit to the Azar *et al.* data. For the Azar *et al.* line baseline PbB was taken to be 16·2 μg/dl, the PbB value associated with a PbA of 0·2 $\mu g/m^3$. For the calculation of α from Kehoe data, for which PbA is not known for the control periods, control PbA was arbitrarily assigned the value 0·2 $\mu g/m^3$. The calculated α values are presented in Table 1.

It is apparent from Fig. 3 that within the range 0–20 μg Pb/m^3 of air, α is a decreasing function of PbA rather than the constant value which would be expected on the basis of a linear model; that is, as ΔPbA increases, α decreases. Values of α calculated from the Griffin *et al.* and Kehoe data points parallel the behaviour of the Azar *et al.* line.

The wide scatter of α values among the Kehoe subjects exposed to $< 10\,\mu g$ Pb/m^3 is due to variation within individuals as well as between individuals. Thus, for example, subject NK (Table 1) had α ranging from 1·00 to 2·94 over the narrow ΔPbA range of 1–3·8 $\mu g/m^3$ (adjusted). Nevertheless, when individual subjects were exposed to a wide range of PbAs, e.g. subjects LD and JOS, there was a clear decrease in α with increasing PbA.

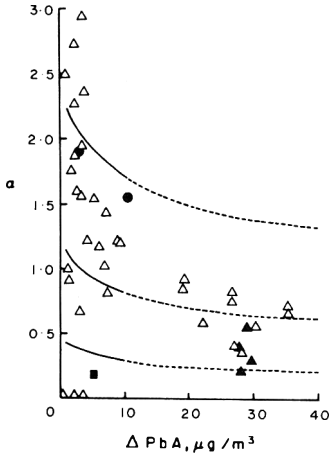

Fig. 3. α as a function of ΔPbA. Data are taken from Table 3. The line of best fit to the Azar *et al.* data and the 95% confidence limits to the line (above and below) are shown dashed beyond the range of actual measurement. Griffin *et al.* mean values (●); Kehoe data points (△); Kehoe data points from individuals exposed to large-particle aerosols (▲); the only positive value of α from subject DH (Kehoe), whose measured PbB from non-air lead sources was exceptionally high (■). The four negative values for Kehoe subjects (see Table 1) have not been plotted.

α is dependent not only on ΔPbA but also on the baseline PbB value; that is on the amount of lead already in the systemic circulation, PbB_0. This dual dependency is illustrated by the simulation in Fig. 4, based on the Azar *et al.* line. Figure 4 shows that the largest calculated values of α should be obtained for groups of subjects with low baseline PbB levels (PbB_0) exposed to small increments in PbA (ΔPbA). In practical terms this behaviour is most marked in the PbB range below about 25 μg/dl, the range associated with ambient air lead levels. When baseline PbB equals or exceeds about 30 μg/dl, α is small, about 0·5 μg/dl for $\Delta PbA = 1{\cdot}0\,\mu g/m^3$, and is affected only slightly if at all by the magnitude of the experimental increment in PbA.

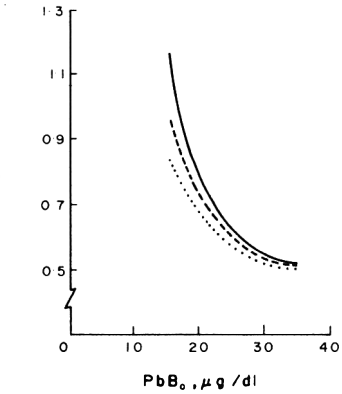

Fig. 4. The relationship of α, simulated using the line of best fit to the Azar *et al.* data, with baseline PbB for three different arbitrary ΔPbAs: $\Delta PbA = 1{\cdot}0\,\mu g/m^3$ (——); $\Delta PbA = 5{\cdot}0\,\mu g/m^3$ (– – –); $\Delta PbA = 10{\cdot}0\,\mu g/m^3$ (· · ·).

The curvilinearity of the PbB–PbA relationship in human subjects is supported by reports of a curvilinear relationship between PbB and drinking-water lead (Moore, Meredith, Campbell, Goldberg & Pocock, 1977), and further evidence comes from related observations in experimental animals. Studies by Azar, Trochimowicz & Maxfield (1973b) and Prpić-Majić, Mueller, Beritic, Stanley & Twiss (1973) suggest that in rats, dogs and rabbits as well as in man, PbB is not directly proportional to lead dose. In the dog and rat studies, the lead salts were added to the diet. In the rabbit study lead acetate was given iv daily for 6 days.

It might be expected that the chemical form and particle size characteristics of lead aerosols would influence absorption rate and efficiency and thereby affect the shape of the observed PbB–PbA curve, made up as it is of data points taken from studies of exposure to air lead having different particle size ranges and chemical makeup. It is unlikely that the chemical form of the lead aerosol is an important factor in determining airway deposition and clearance characteristics, however. Studies by Chamberlain *et al.* (1978) indicate that the chemical form of the lead aerosol generally has little influence on the fractional deposition and clearance of inhaled lead.

On the other hand these studies (Chamberlain *et al.* 1978) have established that the particle size of lead aerosols within the submicron particle size range does influence substantially the degree of deposition in the lungs. This point is illustrated in Fig. 2. Many of the subjects in the Azar *et al.* study were cab drivers exposed while on duty to ambient urban air lead. Chamberlain *et al.* (1978) found the MMED of airborne lead measured either over London streets or in an enclosed London car park to be 0·3 μm. In general the MMED of the lead sesquioxide particles introduced into the Kehoe exposure chambers was 0·26 μm with 90% of the particles having equivalent diameters less than 0·68 μm, but in a few instances large particle aerosols (MMED of up to 3·98 μm) were prepared. [The geometric diameters reported by Kehoe (Gross, 1979) were converted to equivalent diameters for this comparison by using the Stokes-Cunningham equation (NAS-NRC, 1972, Appendix A).] Data points from these experiments are identified as solid triangles in Fig. 2. As would be predicted for exposure to these larger diameter particles, these data points tend to give relatively low α values.

Another identifiable group of four data points, shown as solid squares in Figs 2 and 3 also deviates from the behaviour shown by the others. These are the data from Kehoe subject DH, who had an unusually higher baseline PbB, 30 μg/dl. Their positioning in Fig. 2 as a group below the other Kehoe data points is probably an artefact of the procedure used for baseline adjustment with these data, and is a reflection of the curvilinearity of the PbB–PbA relationship. The ΔPbB associated with a specified ΔPbA is dependent on PbB_0, as discussed above. When PbB_0 is low, ΔPbB for a specified ΔPbA is larger than when PbB_0 is high. Therefore the additive baseline adjustment, while it is the only practical adjustment to use, is not entirely appropriate. It results in a bias in the adjusted PbB data points toward low values. This bias is probably not serious when PbB_0 does not differ greatly from the baseline value of 16 μg/dl, but becomes more serious as PbB_0 increases, so that its effect is clearly apparent in the group of data points from subject DH. Analogous reasoning suggests that the α values calculated for subject DH should be disproportionately low, and indeed three of the four negative calculated α values (Table 1) are associated with subject DH.

It would be of great interest to know how PbB–PbA relationships in children compare with those described here in adults. Unfortunately, there are no data available that would meet or approximate the criteria set forth in the present analysis of the PbB–PbA relationship. In no case has continuous sampling of air lead been done in the breathing zone of children. Only outdoor stationary monitors have been used. Moreover, data in which baseline PbBs were as high as 16 μg/dl are scarce. In one study, however, a comparison was made of the impact of PbA on PbB in adults and children (Johnson, Tillery & Prevost, 1975). Two populations of females and males were compared in which faecal lead excretions were roughly comparable but PbA was 6·3 $\mu g/m^3$ in one case and 0·64 $\mu g/m^3$ in the other. Using the low PbA group as a baseline, α for the high PbA adult males was approximately 1·1 as compared to 1·9 for the male children. In females, α for adults was approximately 0·8 as compared to 0·9 for the children (Johnson *et al.* 1975). Thus, α may be higher for children than for adults, but probably by a factor of less than 2.

It is also interesting to consider the junction of the line of best fit to the Azar *et al.* data with measured PbB–PbA relationships in populations industrially exposed to lead. For many studies of workers no measures of PbB levels in comparable control populations are available, as a result of which the contribution of non-air lead sources is not known. Furthermore, in industrial studies PbA has not always been sampled in the workers' breathing zone. One study in which workers did wear personal air monitors and in which a control population PbB was reported is that of Williams, King & Walford (1969), who studied subjects working in various different departments of a lead-acid battery factory. When the PbB means for the three worker groups are normalized to a control value of 16 μg/dl, as described above for Griffin *et al.* and Kehoe data points, and plotted against the time-weighted average PbA (WHO, 1977), they lie well above the extrapolated line of best fit to the Azar *et al.* data in the PbA range 33–53 $\mu g/m^3$. This discrepancy is unlikely to be attributable to an effect of particle size, since the particular jobs on which the men worked are associated with lead dusts of larger particle size than the lead sesquioxide generated in the Griffin *et al.* and Kehoe studies. It may be at least partly attributable to hand to mouth lead transfer in the worker groups. Williams *et al.* found a significant correlation between personal working habits and total lead exposure within one of their three study groups.

In any case, it should be emphasized that the line of best fit to the Azar *et al.* data presented here represents an empirical fit. As such it cannot be extrapolated beyond the limited concentration range within which it has been shown to be in agreement with

experimentally measured data points; that is, beyond PbA values of about 40 $\mu g/m^3$. It cannot be applied to industrial data, with PbAs as high as 300 $\mu g/m^3$.

In general, significant deviation from linearity of the PbB–PbA relationship as evaluated for industrial populations has not been shown (OSHA, 1978). This observation, which applies to the occupational PbA range 50–300 $\mu g/m^3$, is not inconsistent with the relationship reported here. Figures 2 and 3 show that the curvilinearity of the PbB–PbA relationship diminishes as PbA increases. This behaviour is also reported by Moore *et al.* (1977) in their studies of drinking-water lead.

It is interesting to speculate about the basis for the form of the PbB–PbA relationship. By analogy with observations in animals it seems unlikely that nonlinearities in either absorption or elimination are the primary determinants of the shape of the PbB curve, since if this were the case the effect should be seen in other tissues at about the same exposure level at which it is seen in the blood. In animals, at least, most tissues tend to accumulate lead to a degree much more directly proportional to dose than does the blood. When curvilinearity of the tissue-Pb–PbA relationship is observed, it tends to appear only at exposure levels higher than those associated with the curvature of the PbB–PbA relationship. That the curvilinearity is not a result of nonlinearities in absorption is further supported by its appearance in rabbits administered a lead salt iv (Prpić-Majić *et al.* 1973). It therefore appears most likely that distributional nonlinearities are primarily responsible for the form of the PbB–PbA relationship. It is possible that these nonlinearities are related to the number, kind, and location of lead binding sites in different tissues.

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THE ROLE OF ZINC IN NITRILOTRIACETATE(NTA)-ASSOCIATED RENAL TUBULAR CELL TOXICITY

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Summary—Both the extent and the severity of renal tubular cell toxicity associated with constant systemic loads of nitrilotriacetate (NTA) were shown to be dependent upon the availability of zinc in the circulation. NTA ingestion at doses that produced plasma ultrafiltrate (UF) NTA concentrations $>20\,\mu M$ caused an increase in UF zinc. The majority of the increased zinc but not the NTA was resorbed during renal clearance of NTA and the extent and severity of tubular cell lesions was proportional to the amount of zinc resorbed. Intravenous infusion studies demonstrated that both zinc salts and ZnNaNTA produced nephrotoxicity that is very similar to heavy metal toxicity. The toxicity of iv ZnNaNTA was accompanied by renal tissue accumulation of zinc but not of NTA and the increased tissue zinc was not removed by saline infusion. These studies have led to the development of a model for NTA-associated nephrotoxicity that demonstrates that the initiation, propagation and extent of renal tubular cell toxicity are all dependent upon NTA-induced changes in zinc metabolism. This model negates the use of any mathematical models that estimate low dose ($UF_{NTA} < 20\,\mu M$) effects from toxicity data obtained at doses that exceed the threshold ($UF_{NTA} > 20\,\mu M$).

Introduction

Nitrilotriacetate (NTA) is a divalent metal chelating agent which has been shown to be functional in laundry detergents. Reviews of the mammalian toxicity and environmental effects and fate of NTA have been published (Foley, Becking, Muller, Goyer, Falk & Chernoff, 1977; Shapiro, Chapman, Dick, Dillon, O'Melia, Spacie & Leduc, 1978; Thayer & Kensler, 1973). NTA is readily absorbed from the gut and is excreted only *via* the urine (Michael & Wakim, 1971). In transit through mammals there is no alteration in the structure of the NTA molecule (Thayer & Kensler, 1973) and renal clearance of NTA is accomplished by filtration with no evidence for the existence of a tubular cell transport system for either secretion or resorption (A. Licht, N. S. Bricker, R. E. Papendick & R. L. Anderson, unpublished data, 1980).

NTA ingestion, processing and excretion results in changes in divalent cation (M^{2+}) disposition (Anderson & Kanerva, 1978a; Michael & Wakim, 1973). The divalent cations most sensitive to NTA are magnesium (Mg) and zinc (Zn). NTA increases bone and urinary Zn and decreases faecal Zn (Anderson & Kanerva, 1978a; Nixon, Buehler & Niewenhuis, 1972). In contrast NTA increases faecal Mg and reduces plasma, bone and urinary Mg. Doses of NTA that alter Zn and Mg disposition (0·5% $Na_3NTA.H_2O$ in diet) do not affect calcium (Ca) but higher doses of NTA ($\geqslant$0·75% in the diet) increase urinary Ca excretion (Anderson & Kanerva, 1978b; Anderson & Kanerva, 1979; Michael & Wakim, 1973).

Acute (Merski, 1980), subchronic (Alden & Kanerva, 1979; Mahaffey & Goyer, 1972) and chronic (National Cancer Institute, 1977; Nixon *et al.* 1972) ingestion studies show that the renal tubules are a primary site of NTA-associated toxicity. At dietary NTA doses of up to 18 μmol/g diet (0·5% $Na_3NTA.H_2O$ in the diet) the toxic response noted after 24 months of NTA ingestion has been described as hydropic degeneration (Nixon *et al.* 1972). Higher doses of NTA are associated with renal tubular cell adenoma and adenocarcinoma (National Cancer Institute, 1977). Study of the natural history of NTA-associated tubular cell toxicity from acute, subchronic and chronic studies had led to the conclusion that the process is a continuum, which begins with proximal convoluted tubule (PCT) vacuolization, progresses to simple and nodular hyperplasia with vacuoles to adenomatous hyperplasia, and subsequently appears as tubular cell tumours (Alden, Kanerva, Anderson & Adkins, 1981). In addition to this specific sequence of responses, NTA ingestion also exacerbates the spontaneous nephrosis noted in ageing rats (Alden & Kanerva, 1980). The PCT toxicity, even at the nodular hyperplasia stage is reversible if NTA is removed from the diet (M. C. Myers, R. L. Kanerva, C. L. Alden & R. L. Anderson, unpublished data, 1980).

Extensive testing has shown that NTA is not mutagenic or teratogenic (Foley *et al.* 1977). This finding strongly implies that the tumorigenicity associated with the ingestion of high doses of NTA is an epigenetic phenomenon. Thus the primary concern is how a molecule that is devoid of genotoxicity, is not metabolized to a genotoxic moiety, is not transported across PCT cells, and the only significant chemical activity of which is its propensity to form stable complexes with M^{2+}, can be responsible for the PCT toxicity (including tumours) noted after chronic exposure at high doses.

A comparison of the dose-response curves showed a very close similarity between PCT toxicity and increased Zn excretion in the urine. For example, 24 months of ingesting a diet containing 1·1 μmol NTA/g

(0·03% $Na_3NTA.H_2O$) caused no alteration in renal morphology or urinary Zn excretion. Dietary NTA at 5·5 μmol/g (0·15% $Na_3NTA.H_2O$) increased urinary Zn excretion and was associated with PCT vacuoles and hyperplasia, changes described as hydropic degeneration (Nixon *et al.* 1972). Both urinary Zn excretion and PCT lesions were enhanced when dietary NTA was increased to 18 μmol/g (0·5% $Na_3NTA.H_2O$ and 0·5% CaNaNTA).

This report describes the results of a series of experiments in which the relationship between NTA-induced changes in Zn disposition and PCT toxicity was more extensively examined. The results of these experiments show that the initiation of PCT toxicity is dependent upon blood NTA attaining concentrations high enough to increase the plasma ultrafiltrate Zn (UF_{Zn}) levels. A large proportion of the increased UF_{Zn} is resorbed by the PCT during renal clearance of NTA, and by some undefined mechanism this alters the endocytotic-lysosomal system to induce vacuoles and subsequently tubular cell hyperplasia. This response has demonstrable no-effect levels which negate the validity of mathematical extrapolations of responses (including tumours) noted at high doses to possible incidences at low doses.

Experimental

All of the experiments reported used Sprague–Dawley-derived rats obtained from Charles River Breeding Laboratories, Inc., Wilmington, MA. The animals were housed individually in stainless-steel metabolism cages and kept at controlled temperature (70 ± 5°F) and humidity (40–60%) with a 12-hr light/dark cycle. In most of the experiments the diet was ground Purina Laboratory Chow (Ralston Purina Co., St Louis, MO) and the animals were given feed and distilled water *ad lib.* For one study the rats were trained to consume their daily intake of feed in a 1-hr period (9–10 a.m.). Meal training was accomplished by reducing the time during which the diet was available over a 2-wk period. In the study in which the dietary Zn level was varied the animals were fed a semi-purified diet containing 8, 14, 21 or 52 ppm Zn. The diet was composed of 20% casein, 20% lard, 60% sucrose and all of the vitamins and minerals (except Zn) at greater than the recommended daily allowance (National Academy of Sciences/National Research Council, 1972) for the rat. In all instances NTA was added to the diets on a w/w basis. Weekly weight gains and twice-weekly feed consumption records were maintained on all animals. Urine samples for mineral and NTA analyses were total 24-hr collections obtained in cages with a separator which retained the faeces and allowed urine to be collected in tared bottles. The separators were not rinsed.

For iv infusions, 200–250 g male rats were fitted with catheters of Silastic 602-135 tubing (Dow Corning Corp., Medical Products, Midland, MI) in the jugular vein as described by Upton (1975). The catheters were connected to a Harvard 975 Infusion Pump (Harvard Apparatus, Millis, MA) set to deliver 10 μl/min. The animals were restrained throughout the infusion period and were provided with chow pellets (Ralston Purina Co.) and distilled water *ad lib.* During infusion the voided faeces were collected on a nylon screen over a funnel and the urine was collected in tared vials.

Before they were killed the animals were anaesthetized with ether, and then a mid-line incision was made and blood was collected from the vena cava in heparinized syringes. The blood cells were separated by centrifugation and when required, plasma ultrafiltrate (UF; MW < 10,000) was obtained by filtering a portion of the plasma through an Amicon PM-10 filter (Amicon Corp., Lexington, MA). Minerals were determined by atomic absorption spectrometry. Urine NTA analyses were by reverse isotope dilution when unlabelled NTA was fed and by direct count after 25:1 dilution with water when [^{14}C]NTA was used (Anderson & Kanerva, 1978b). Blood-cell, plasma and UF [^{14}C]NTA analyses were carried out after combustion of [^{14}C]NTA to $^{14}CO_2$. All ^{14}C levels were determined by scintillation counting.

The kidneys were processed using routine histological techniques followed at our laboratory. The tissue was placed in 10% neutral phosphate-buffered formalin and allowed to fix for 18 hr. The tissues were embedded in paraffin blocks according to standard histological methods, sectioned on a microtome at 5 μm, stained with haematoxylin and eosin and evaluated by light microscopy. [For further details see Alden *et al.* (1981) and Merski (1981).] Electron microscopy was carried out by Mr M. Myers of this laboratory.

All forms of unlabelled NTA were prepared from a commercial sample of $Na_3NTA.H_2O$ (Monsanto Co., Alvin, TX). H_3NTA was prepared by treating solutions of $Na_3NTA.H_2O$ with concentrated HCl which precipitates the H_3NTA. The divalent metal (M^{2+}) complexes of NTA were prepared by stirring equal molar amounts of the desired metal oxide and H_3NTA in distilled water for about 1 hr and then titrating to a pH of 7·4 with NaOH (NH_4OH was used for the Ca complex). The complexes were diluted to the desired concentrations with water. Each complex was assayed for its M^{2+} content to define the NTA complex concentration for infusion. The ZnNaNTA for feeding was prepared in the same way but was precipitated by addition of three volumes of 95% (v/v) ethanol and dried in a vacuum oven at 30°C. The [^{14}C]NTA used in all cases was labelled in the carboxyl groups of the acetate units. Periodic determinations of [^{14}C]NTA stability were made by isotope dilution with H_3NTA.

Results

The correlation between NTA-associated PCT toxicity and increased urinary Zn excretion suggested that ingestion of the Zn complex of NTA (ZnNaNTA) could result in increased NTA renal toxicity. To test this hypothesis, mature male rats were fed diets containing 0·5, 1·0, 1·5 or 2·0% ZnNaNTA for 3 wk. After 21 days of *ad lib.* feeding, the levels of Zn and NTA in a 24-hr urine collection were measured, and the kidney:body weight ratio was ascertained as an indicator of renal toxicity (Anderson & Kanerva, 1978a). The urinary NTA recovery was low and approximately constant at the three lowest ingestion rates but was increased at the highest intake rate (Fig. 1a). Urinary Zn displayed a linear increase with ingestion rate

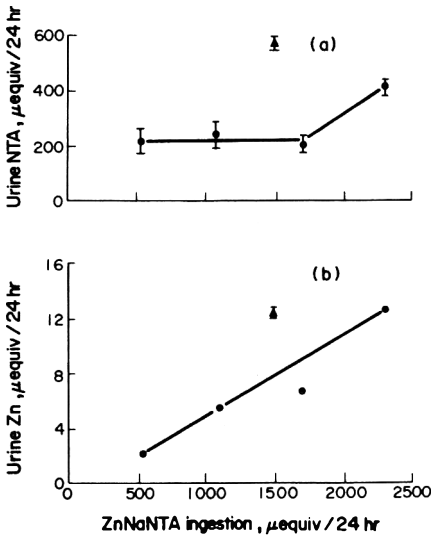

Fig. 1. Effect of ingestion of ZnNaNTA (●) at dietary levels of 0, 0·5, 1·0, 1·5 or 2·0% for 3 wk on (a) urinary NTA levels (mean ± SEM for five rats) and (b) urinary Zn levels (single analysis on pooled urine from five rats) in adult male rats. For comparison, the effect on the same parameters of $Na_3NTA.H_2O$ given at 2·0% in the diet is also shown (▲; mean ±SEM for five rats).

(Fig. 1b) but it did not approach the urinary NTA concentration. This clearly shows that the ZnNaNTA complex does not remain intact during passage through the body. The kidney:body weight ratio was not significantly influenced by any of the ZnNaNTA doses.

For comparative purposes the effect of the highest dietary level of NTA (2%) as its trisodium salt ($Na_3NTA.H_2O$) on the same parameters is included in Fig. 1. $Na_3NTA.H_2O$ ingestion resulted in about a three-fold greater urinary NTA load and a marked increase in kidney:body weight ratio (data not shown) and an even higher urinary Zn level at a much lower NTA intake level. This suggests that if NTA-induced renal toxicity is causally related to altered Zn metabolism the effects are due to NTA-associated alterations in systemic Zn metabolism and are not a consequence of ingested ZnNaNTA absorption and excretion in the urine. This contention is supported by the results of a study of the effect of NTA on cation balance (Anderson & Kanerva, 1978a), which demonstrated that the increased urinary Zn excretion associated with NTA ingestion was compensated for by decreased faecal Zn excretion, so that NTA did not alter Zn balance compared to that of control rats.

Since increasing dietary Zn had the net effect of reducing NTA absorption, the renal toxicity associated with lowered dietary Zn at a constant dietary NTA level was ascertained. Male rats were fed semi-purified diets containing 1·2% $Na_3NTA.H_2O$ with either 52 (equivalent to chow), 21, 14, or 8 μg Zn/g diet. After 4 wk of ingestion of these diets *ad lib.*, plasma-Zn and urinary Zn and NTA levels were measured and the kidneys were evaluated histologically with emphasis on the specific NTA-associated histopathology (vacuoles and hyperplasia in the PCT). At the three lowest dietary Zn levels NTA administration resulted in a reduced plasma-Zn concentration which was overcome at the highest dietary Zn level (Fig. 2a). In contrast, at all dietary Zn levels, NTA ingestion resulted in an increase in urinary Zn excretion relative to control rats (Fig. 2b). This increase was dependent on dietary Zn concentration. The urinary NTA level was approximately constant at all four dietary Zn levels (Fig. 2b). Thus the aim of establishing approximately constant urinary NTA excretion with markedly different Zn excretion was achieved. In addition, these results show that when dietary Zn is limited, the increased urinary Zn excretion associated with NTA excretion results in decreased plasma-Zn levels.

Table 1 summarizes the results of the histopathology noted in the kidneys from the animals fed the two highest doses of Zn, expressed as the toxicity index, that is the product of incidence (kidneys having the specific lesion type/ten kidneys examined) and frequency (average number of the specific lesion/kidney section). The toxicity indices for the kidneys from rats fed the two lowest levels of Zn (8 and 14 ppm) are not included in Table 1 since in the 14 sections examined only vacuolated tubules and two tubules with

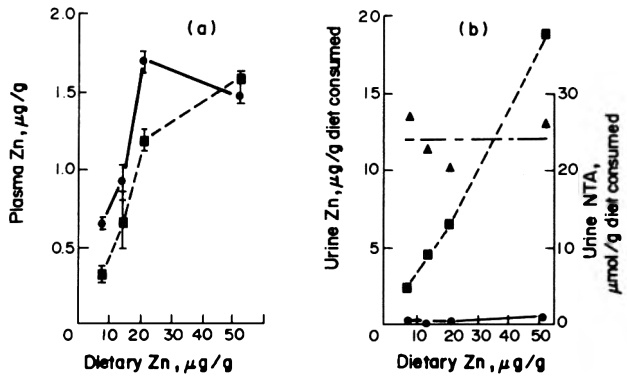

Fig. 2. Effects of different levels of dietary Zn (8, 14, 21 or 52 ppm) on (a) plasma Zn and (b) urine Zn in rats given either 0 (●——●) or 1·2% (■- - - -■) $Na_3NTA.H_2O$ in the diet. Urinary NTA levels are also shown (▲——▲). Plasma values are means ±SEM for groups of five rats. The urine values were obtained on a pooled 24-hr urine sample from five rats per treatment.

Table 1. *Effect of dietary zinc concentration on renal tubular cell damage in male rats fed semi-purified diets containing 1·2% $Na_3NTA.H_2O$ for 4 wk*

Tubular cell damage	Toxicity index* for rats given 21 ppm Zn	Toxicity index* for rats given 52 ppm Zn
Vacuolization and simple hyperplasia	15 (0)†	25 (0)
Vacuolization and nodular hyperplasia	3 (0)	50 (0)

*Toxicity index = incidence (number of kidney sections showing lesion/treatment group) × frequency (mean number of tubules showing lesion/kidney section). Ten kidneys were examined in each NTA-treated and control group.

†Values in brackets are for control rats given diets that did not contain $Na_3NTA.H_2O$.

vacuoles and simple hyperplasia were noted. Thus low dietary levels of Zn resulted in minimal PCT toxicity. The zero incidence for the two types of lesions in the control groups confirms that the lesions noted are specific responses to NTA. The PCT toxicity index in the rats fed the diet containing 52 ppm Zn was greater for both stages of toxicity development than in the rats fed the diet containing 21 ppm Zn. The effect of dietary Zn on the PCT toxicity was particularly evident with respect to the more severe lesion (PCTs with vacuoles and nodular hyperplasia), for which the toxicity index in the group ingesting the diet with 21 ppm Zn was only three compared to a value of 50 for the group given 52 ppm Zn.

These feeding studies demonstrate that the NTA-specific toxicity in the renal PCT, assessed after comparable levels of NTA have been excreted in the urine, is proportional to the amount of Zn but independent of the amount of NTA that has been excreted in the urine. This suggests that Zn availability in the circulation is a limiting factor in NTA-induced PCT toxicity. This hypothesis was further tested by ascertaining the effect of Zn administered by iv injection ($ZnSO_4$ at 0·3 mmol/kg given 30 min after NTA) on the response of PCTs to an acute oral dose of NTA (7·3 mmol/kg of $Na_3NTA.H_2O$ titrated to pH 8·5 with HCl). The number of PCTs containing vacuoles was markedly increased in the animals given iv Zn after the NTA dose compared to those only given the oral NTA (Plate Ia,b). No PCT vacuoles were noted in kidneys from rats given iv Zn but no NTA. Thus both the acute (PCT vacuoles) and the subchronic nephrotoxicity (PCTs with vacuoles and hyperplasia) associated with a constant NTA dose are proportional to available Zn in the circulation.

The fact that renal clearance of NTA is *via* filtration with no evidence of a tubular cell transport system for NTA (Licht *et al.* 1980), coupled with the established relationship of PCT toxicity to Zn availability, prompted a detailed examination of the effects of NTA on Zn disposition and excretion. The effects of a nephrotoxic (73 μmol/g diet), a non-nephrotoxic (0·73 μmol/g diet) and an intermediate (7·3 μmol/g diet) dose of [^{14}C]NTA on Zn metabolism were compared in rats equilibrated with [^{14}C]NTA by 10 days of *ad lib.* ingestion. [^{14}C]NTA and Zn levels were measured in the plasma UF and a 24-hr voided urine sample to determine if renal clearance of NTA resulted in a change in the NTA:Zn ratio in the urine relative to that in its precursor, plasma UF. A lower NTA:Zn ratio in the urine relative to that in the UF would show an NTA-induced Zn secretion, whereas a higher ratio in the urine than in the UF would show specific Zn resorption. For reference, in control rats the majority of the filtered Zn is resorbed by tubular tissue (Yunice, King, Kraikitpanitch, Haygood & Lindeman, 1978). The two lower doses of NTA resulted in similar UF_{Zn} levels but the highest NTA dose produced a five-fold increase in UF_{Zn} levels (Table 2). At all of the NTA doses, the NTA:Zn ratio in the urine (product) was increased over the ratio in the UF (precursor) showing net renal resorption of the Zn. A constant proportion of the filtered Zn was resorbed since in each instance the NTA:Zn ratio in the urine was 2–3 times that in the UF. The mass of Zn resorbed (estimated from the UF NTA:Zn ratio and the urine total NTA and Zn levels) was only 0·4 or 1·7 μmol/day following ingestion of 0·73 or 7·3 μmol NTA/g diet, respectively, but there was a twelve-fold increase in Zn resorption (11·9 μmol/day) at the nephrotoxic dose of NTA (73 μmol/g diet). This suggests that PCT toxicity is likely to be a consequence of tubular cell Zn resorption.

To determine the dose of NTA required to increase UF_{Zn}, rats trained to consume their daily intake of diet in a 1-hr period were used to measure UF_{NTA} and UF_{Zn} levels at the time of maximum blood-NTA levels (4 hr after the meal). Figure 3 shows that NTA doses that resulted in $UF_{NTA} \leqslant 21$ μM did not alter the UF_{Zn} level but that the higher UF_{NTA} level (52 μM) was accompanied by an increased UF_{Zn} level. Thus ingestion of high doses of NTA induces an increase in plasma UF_{Zn} concentration that is largely resorbed during passage through the kidney. Further, the plasma UF_{NTA} level must exceed a threshold of about 20 μM in order to induce an elevation in UF_{Zn} levels.

Table 2. *Effect of dietary NTA concentration on plasma ultrafiltrate (UF) and urinary concentrations of NTA and zinc*

Dietary NTA (μmol/g diet)	Concentration (nequiv/ml) of NTA	Concentration (nequiv/ml) of Zn	Ratio NTA:Zn
	Plasma UF		
0·73	1·41 ± 0·10	0·79 ± 0·45	2
7·3	11·9 ± 0·6	0·77 ± 0·18	15
73	119 ± 11	3·8 ± 0·70	31
	Urine		
0·73	0·30 ± 0·04	0·06 ± 0·01	5
7·3	2·77 ± 0·39	0·07 ± 0·01	40
73	27·2 ± 3·8	0·25 ± 0·05	109

Each value for UF and urinary Zn and NTA is the mean ± SEM for groups of 12 rats (6 males and 6 females). Three rats of each sex were fed the indicated dietary concentration of either H_3[^{14}C]NTA or Na_3[^{14}C]NTA.H_2O *ad lib.* for 10 days. The urine values are based on a total 24-hr urine collection and the UF samples were obtained between 8 and 10 a.m.

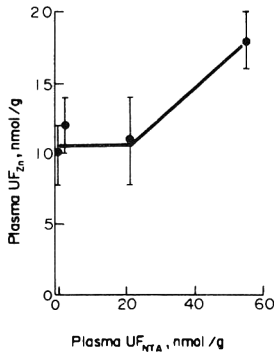

Fig. 3. Effect of plasma ultrafiltrate NTA levels (UF_{NTA}) on plasma ultrafiltrate Zn levels (UF_{Zn}) in rats ingesting their NTA dose in a 1-hr period. Blood was obtained 4 hr after the tenth NTA meal. Each value is the mean ±SEM for three samples.

In the experiments described, the total-plasma and plasma-UF concentrations of Ca and Mg were also measured. NTA ingestion did not alter total-plasma Ca or UF_{Ca} levels in any study but it did reduce total-plasma Mg and UF_{Mg} levels at the time of maximum levels of blood NTA (4 hr after the meal). The reduced Mg levels probably reflect changes in Mg absorption since NTA ingestion increases faecal Mg (Anderson & Kanerva, 1978a).

To examine further the role of Zn in the PCT toxicity associated with renal clearance of NTA, the effect of iv infusions of the forms of NTA most likely to be present in the plasma were compared. The iv dose chosen (6 mmol/kg/24 hr, except for ZnNaNTA) was equivalent to twice the systemic load of NTA in animals consuming diets containing 73 μmol/g. Five forms of NTA were examined—the Zn, Ca and Mg complexes and the Na and K salts. The infusions with the two salts and the Ca and Mg complexes of NTA at 6 mmol/kg/24 hr for 3–7 days all induced some PCT vacuoles (Plate II). These infusion studies resulted in no more PCT toxicity than had been noted after 3 days of ingestion of a diet delivering approximately one-half the systemic dose of NTA (Plate III). In contrast, infusion of the ZnNaNTA at its limiting solubility of 3 mmol/kg/day was lethal to every animal in less than 48 hr. The animals displayed a very consistent response during ZnNaNTA infusion; for the 0–24-hr period the animals all voided urines containing glucose, protein and haemoglobin. At about 24 hr the animals ceased to urinate and soon became comatose. Kidneys removed after 48 hr of ZnNaNTA infusion at 3 mmol/kg/24 hr had a speckled surface and when sectioned showed a distinct white precipitate at the cortico-medullary junction. Histologically, the kidneys displayed massive renal coagulative necrosis and tubular cell sloughing. Electron-microscope evaluation of the material accumulated at the cortico-medullary junction showed sloughed tubular cells and intra- and intercellular crystalline material.

The recovery of [^{14}C]NTA in the urine following iv infusion of various ^{14}C-labelled forms of NTA showed that with all NTA species examined, including ZnNaNTA, more than 90% of the infused [^{14}C]NTA was recovered in the urine. The high and constant urinary recovery of NTA, regardless of NTA species infused, indicated that the nephrotoxicity induced by ZnNaNTA was a consequence of the Zn and not of increased retention of NTA. This contention was further investigated by comparing the renal toxicity of Zn salts and Zn ethylenediaminetetraacetic acid (ZnEDTA), a more stable complex than ZnNaNTA. When infused at 3 mmol/kg/day, the Zn salts produced the same *in vivo* response as ZnNaNTA but in a shorter time period, and also caused PCT necrosis. In contrast, ZnEDTA infused at 3 mmol/kg/day for 3 days produced only minimal renal damage (a few PCT vacuoles).

Comparison of urinary recovery of the Zn when given as an infusion of ZnNaNTA, ZnEDTA, Zn acetate or Zn gluconate (Fig. 4) showed that the renal toxicity induced was inversely proportional to the urinary recovery of the infused Zn and, therefore, proportional to the carcass retention of the infused Zn. The renal toxicity induced by iv Zn (necrosis and cell sloughing) was different from that associated with NTA ingestion (vacuoles and hyperplasia). However, when the iv dose of ZnNaNTA was reduced to *c.* 1 μmol/rat/day, the PCT response was similar to that induced by dietary NTA (Plate IV).

In order to contrast the acute effects of ZnNaNTA infused iv with those of dietary NTA, the total-plasma, plasma UF and urinary [^{14}C]NTA:Zn ratios were determined after 72 hr of infusion with ZnNa[^{14}C]NTA. These parameters showed that at least after 48 hr of infusion the Zn and NTA were distributed differently in the blood pools than in the infusion solution (Table 3). The blood cells and plasma proteins contained more Zn than NTA. In contrast the plasma UF contained far more [^{14}C]NTA than Zn. This was accompanied by a urinary NTA:Zn ratio that was lower than the NTA:Zn ratio in the UF—a result opposite from that noted in rats fed NTA (Table 2). Thus ZnNaNTA infused iv dissociates in the circulation resulting in a marked elevation in plasma-protein and cellular Zn and Zn secretion into the urine during NTA clearance. This implies that the difference in renal lesions between ZnNaNTA infused iv and dietary NTA could be due to either or both of the following: (1) a difference in the route of Zn entry into the tubular cell—from

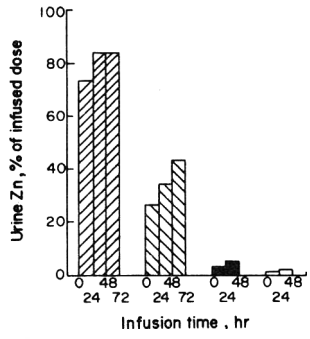

Fig. 4. Comparison of urinary recovery of Zn during iv infusion of ZnEDTA (▨), ZnNaNTA (▩), Zn acetate (■) or Zn gluconate (□) at levels to give 7·3, 7·6, 7·7 or 7·4 mg Zn/24 hr, respectively.

Table 3. *Blood component and urine NTA and Zn levels after 48 hr of iv infusion of ZnNaNTA*

Fluid or tissue	Conc (μM) NTA	Conc (μM) Zn	Ratio NTA:Zn
Rats given 4·2 μmol ZnNaNTA/hr			
Infusion fluid	7100	7000	1·01
Blood cells	3·2 ± 0·5	292 ± 25	0·011
Plasma	26 ± 3	159 ± 4	0·164
Plasma ultrafiltrate	19 ± 2	1·3 ± 0·7	14·6
Urine	2600 ± 300	1000 ± 200	2·5 ± 0·5
Rats given 12·6 μmol ZnNaNTA/hr			
Infusion fluid	21400	21000	1·02
Blood cells	20 ± 1	1159 ± 103	0·017
Plasma	85 ± 7	407 ± 20	0·209
Plasma ultrafiltrate	56 ± 5	7·4 ± 2·0	7·6
Urine	10200 ± 600	6400 ± 400	1·6 ± 0·1

Values are means ± SEM for groups of three male rats (*c.* 250 g) infused iv with the indicated dose of ZnNa[^{14}C]NTA for 48 hr. The infusion volume was *c.* 10 μl/min.

the circulation into tubular cells with infused ZnNaNTA and from the tubular lumen into PCT cells with dietary NTA; (2) differences in the Zn concentration obtained in the circulation—dietary NTA does not appreciably increase Zn levels in the total plasma but does shift the distribution from the plasma-protein pool (MW > 10,000) to the UF while iv ZnNaNTA causes a marked increase in Zn in the total-plasma pool, but most of the Zn is protein-bound. The fact that infusion of Zn salts produces a response similar to ZnNaNTA suggests that the renal tubular necrosis is associated with the increase in plasma-protein Zn levels since very little of the infused Zn from the salts enters the urine. Further, infusion of Zn gluconate results in a dose-dependent increase in renal-tissue Zn which is readily removed by short-term infusion of saline after Zn gluconate infusion (Fig. 5).

The results with iv-infused Zn (both as the NTA complex and as salts) are indicative of acute heavy-metal toxicity (Hammond & Belites, 1980). This similarity was confirmed by showing that $HgCl_2$ infused iv (21 μmol/rat for 4 hr) resulted in the same series of *in vivo* responses with iv Zn, and histological evaluation of the kidneys from these animals showed marked tubular cell necrosis (Plate V).

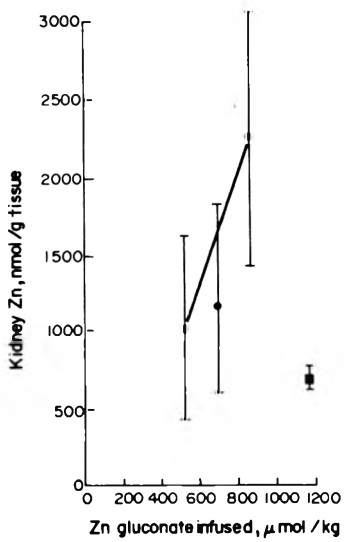

Fig. 5. Renal tissue Zn concentration as a function of the amount of Zn gluconate infused iv: with only Zn gluconate infusion (●); with Zn gluconate infusion followed by a 4-hr saline infusion (■). Values are means ± SEM for groups of three rats.

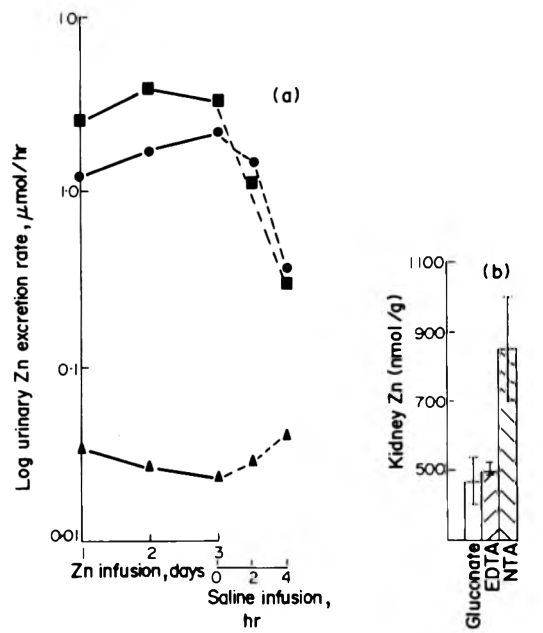

Fig. 6. (a) Urinary Zn excretion rate during 3 days of iv infusion (——) of Zn gluconate (▲), ZnNaNTA (●) or ZnEDTA (■) at *c.* 550 μmol/kg/day and for two subsequent 2-hr periods of saline infusion (– – –). (b) Levels of Zn in kidney after 4 hr of saline infusion following Zn gluconate (□), ZnEDTA (▨) or ZnNaNTA (▩) infusion. Renal zinc values are means ± SEM for groups of two rats. Rats infused with ZnEDTA, ZnNaNTA and Zn gluconate excreted, respectively, 72, 37 and < 1% of the infused zinc in their urine.

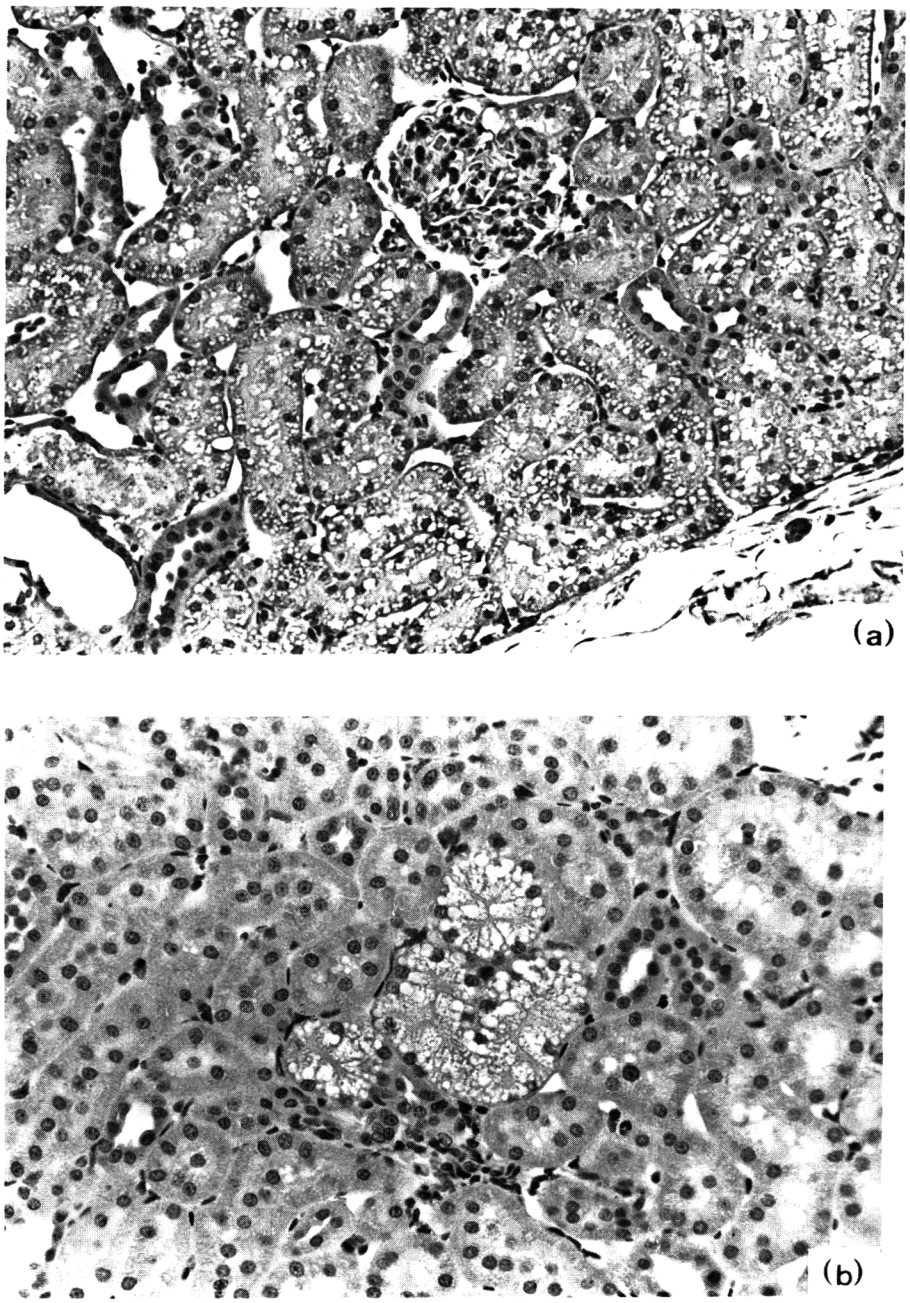

Plate I. Photomicrographs of kidneys from rats 6 hr after a gavage of 7·3 mmol NTA/kg body weight; (a) kidney of a rat given iv 0·3 mmol $ZnSO_4$/kg body weight 30 min after the NTA gavage; (b) kidney of a rat given iv saline after oral NTA. Haematoxylin and eosin × 130.

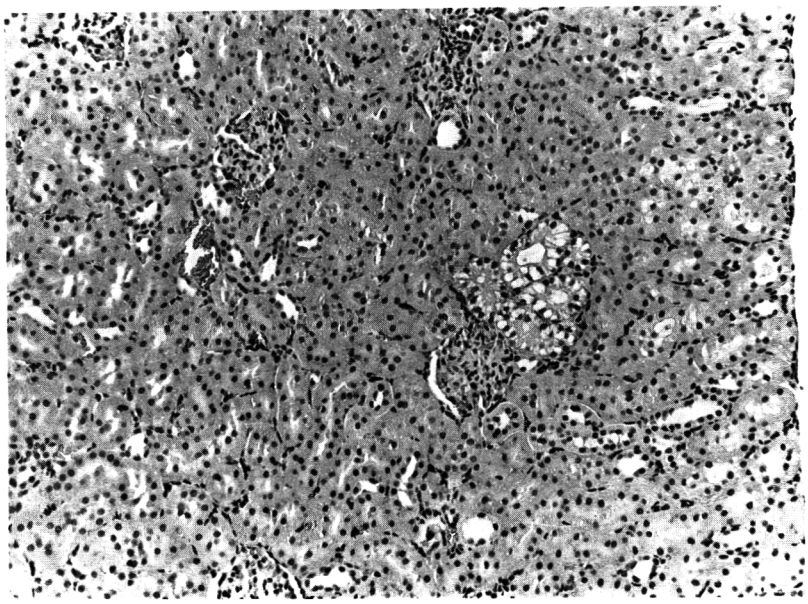

Plate II. Photomicrograph of the kidney of a rat infused for 72 hr with 6 mmol NTA/day. The photomicrograph is from an animal infused with MgNaNTA but similar effects were induced in the kidneys of animals infused with $Na_3NTA.H_2O$, $K_3NTA.H_2O$ or CaNaNTA at the same exposure rate. Haematoxylin and eosin × 70.

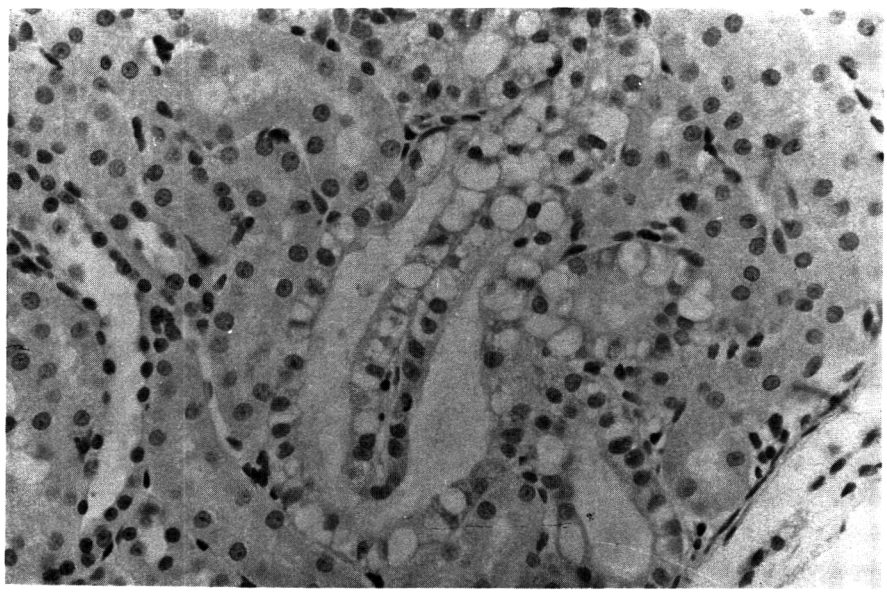

Plate III. Kidney section from a rat fed a diet containing 2% $Na_3NTA.H_2O$ for 3 days. Note the focus of tubules with vacuoles and hyperplasia. Haematoxylin and eosin × 350.

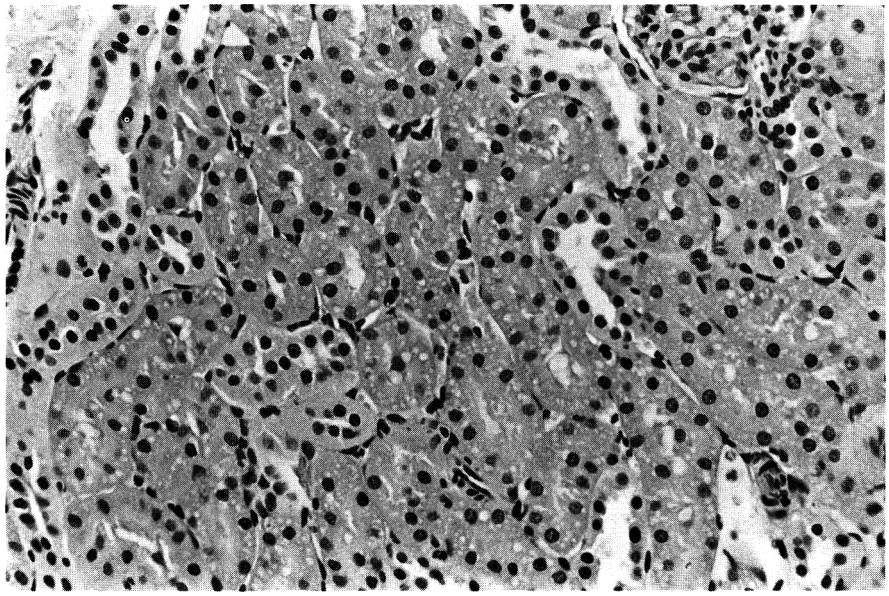

Plate IV. Photomicrograph of the kidney from a rat infused with 2·25 μmol ZnNaNTA/day for 2 days. Note proximal convoluted tubule with vacuoles. Haematoxylin and eosin × 130.

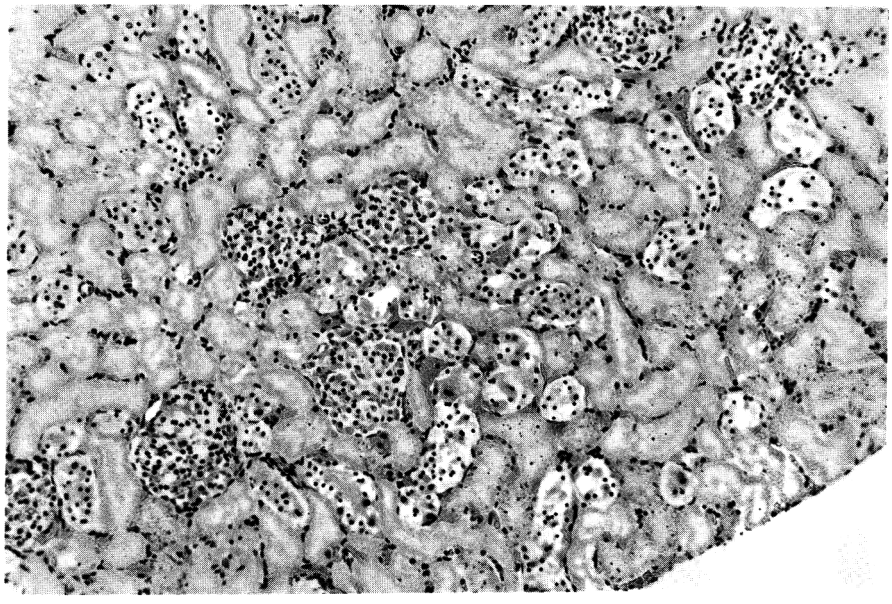

Plate V. Photomicrograph of a kidney section of a rat infused iv with 125 μmol $HgCl_2$/kg/24 hr for 4 hr. Haematoxylin and eosin × 70.

Table 4. *Kidney Zn and NTA concentrations after ZnNaNTA infusions*

Parameter	Results following ZnNaNTA infusion at (μmol/kg/day)	
	400	1200
Kidney excess Zn*(mmol/g)	240 ± 21	1020 ± 65
Kidney NTA (mmol/g)	174 ± 22	588 ± 110
Kidney Zn:NTA	1·43 ± 0·26	1·81 ± 0·22
Urine Zn:NTA	0·33 ± 0·04	0·62 ± 0·04

*Kidney Zn in rats infused with ZnNaNTA minus kidney Zn in rats infused with saline.

Each value is the mean ± SEM for samples from three rats infused with the indicated level of ZnNaNTA for 3 days.

To further differentiate between the renal effects of ZnNaNTA, Zn gluconate and ZnEDTA, the renal Zn levels were determined after 3 days of iv infusion of each of these forms of Zn at *c.* 550 μmol/kg/day. To minimize tissue contamination with entrapped urine Zn, the animals were infused with saline for 4 hr before they were killed. Figure 6 shows that the Zn level in the kidneys from the rats infused with Zn gluconate and ZnEDTA were comparable even though the former excreted $<1\%$ of the infused Zn in their urine and the latter excreted $>70\%$. In contrast, the kidney Zn level after infusion with ZnNaNTA was about 100% greater than that after infusion with Zn gluconate or ZnEDTA. This increased kidney Zn level could not be attributed to a higher urinary Zn concentration at the time of death compared with that of rats infused with ZnEDTA. Thus NTA results in a specific renal tissue Zn accumulation that is resistant to washout and that is not simply the result of either increased systemic Zn (as brought about by Zn gluconate) or increased urinary Zn excretion (as caused by ZnEDTA). To determine if the NTA-associated renal accumulation of Zn was due to ZnNaNTA accumulation, the excess kidney Zn (i.e. levels in ZnNaNTA-infused kidneys − levels in saline-infused kidneys) and [^{14}C]NTA concentrations were ascertained after 48 hr of iv infusion of ZnNa[^{14}C]NTA at 400 or 1200 μmol/kg/day. At both ZnNaNTA infusion rates there was a greater excess kidney Zn than [^{14}C]NTA (Table 4). Further, since the urine contained more NTA than Zn the increased tissue Zn cannot be attributed entirely to urine contamination. To place the tissue NTA levels in perspective the entrappment of 59 ± 6 μl urine/g tissue could account for all of the kidney NTA at both doses but for only 70 and 400 nmol Zn/g tissue at the low and high doses, respectively. Thus not all of the renal tissue Zn that accumulates during NTA clearance is derived from ZnNaNTA. This becomes even more significant when it is recalled that in feeding studies it was shown that renal clearance of NTA was accompanied by a net Zn resorption.

Discussion

The results of the experiments reported show that the extent of damage induced in the renal PCT during clearance of constant amounts of NTA is directly proportional to the availability of Zn in the circulation. Both the induction of vacuoles in PCT cells and the subsequent development of hyperplasia in these cells, at a constant level of NTA excretion, are proportional to the availability of Zn in the circulation. In addition, the results show that Zn infused iv is a renal toxin in the absence of NTA when it is retained in the carcass (Zn salts) but its toxicity is markedly reduced when it is excreted in the urine as a complex (ZnEDTA). In this sense Zn nephrotoxicity is similar to mercury nephrotoxicity—mercury salts are potent nephrotoxins but mercurial diuretics are not (for discussion see Zbinden, 1971).

Examination of the effects of NTA on Zn disposition shows that when the UF_{NTA} attains sufficient concentration (>20 μM) it increases the concentration of UF_{Zn}. During renal processing of the UF fraction a large portion of the Zn, but little, if any, of the NTA is resorbed. The increase in Zn resorption is a necessary condition for the initiation of PCT toxicity, and since the initial PCT toxicity is clearly a necessary but not sufficient condition for eventual renal tumorigenesis (Alden *et al.* 1980), doses of NTA that do not initiate the response will not increase the renal tumour incidence relative to that of controls. The demonstration of a distinct threshold for an NTA effect on PCT toxicity certainly negates the validity of any mathematical model for extrapolation of possible toxicity including tumorigenicity at a dose below this threshold from results noted at doses that clearly exceed this threshold. The results noted in chronic ingestion studies are in agreement with this model in that only doses of NTA that increase urinary Zn have resulted in any renal tubular cell lesions and only extreme doses have been associated with renal tubular cell neoplasia (National Cancer Institute, 1977; Nixon *et al.* 1972). In contrast, chronic exposure to NTA doses that do not alter dietary Zn disposition ($\leqslant 0{\cdot}03\%$ $Na_3NTA.H_2O$) do not induce any renal toxicity even after 24 months of ingestion (National Cancer Institute 1977; Nixon *et al.* 1972).

Finally, a calculation of the average exposure of man to NTA from drinking-water, based on measured values in Canada, where NTA has been used in detergents since 1970 (Malaiyandi, Williams & O'Grady, 1979), shows an average exposure of 0·1 μg/kg/day (35 ml H_2O/kg/day × 2·82 μg NTA/10^3 ml H_2O). If all of this daily ingested dose of NTA were in the plasma UF pool at one time it would amount to only 0·36 nequiv of NTA/kg or 0·009 μM (assuming 40 ml UF/kg), more than three orders of magnitude less than the plasma UF_{NTA} threshold of >20 μM which is

necessary to increase the UF_{Zn} level and initiate PCT toxicity. In reality, man shows low absorption of ingested NTA (*c.* 12%) and rapid urinary clearance (Budny & Arnold, 1973) so that the ingestion of 0·1 μg/kg/day would result in UF_{NTA} several orders of magnitude less than the UF_{NTA} threshold established in rats.

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FAILURE OF CHLOROFORM TO INDUCE CHROMOSOME DAMAGE OR SISTER-CHROMATID EXCHANGES IN CULTURED HUMAN LYMPHOCYTES AND FAILURE TO INDUCE REVERSION IN *ESCHERICHIA COLI*

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Summary—Two strains of *Escherichia coli*, WP2p and WP2uvrA⁻p, were treated with chloroform in the plate incorporation assay, and in liquid pre-incubation tests, and in both cases the chemical did not induce reversions. Chloroform, with metabolic activation (addition of S-9 mix), also failed to induce chromosome breakage or sister-chromatid exchanges in human lymphocytes, but benzo[*a*]pyrene, with and without S-9 mix, induced significant chromosome breakage and sister-chromatid exchanges in lymphocytes from the same donor. The relevance of these results in the light of other negative *in vitro* tests on chloroform, and positive and negative carcinogenicity tests, is discussed.

Introduction

Chloroform has been widely used over the last century as an anaesthetic, solvent, preservative and flavour additive, and an evaluation of the safety to humans of such a ubiquitous chemical is clearly important. Eschenbrenner & Miller (1945) reported induction of hepatomas in female Strain A mice after repeated oral administration of chloroform in olive-oil solution, but only when the doses were large enough to produce liver necrosis. The results of a study carried out by the National Cancer Institute (1976) on chloroform, given as a solution in corn oil, indicated a significant increase in kidney epithelial tumours in male Osborne–Mendel rats, and a significant increase in hepatocellular carcinomas in male and female $B6C3F_1$ mice. However, long-term administration of chloroform in a toothpaste vehicle to mice (Roe, Palmer, Worden & Van Abbé, 1979), rats (Palmer, Street, Roe, Worden & Van Abbé, 1979) and dogs (Heywood, Sortwell, Noel, Street, Prentice, Roe, Wadsworth, Worden & Van Abbé, 1979) produced no significant increase in neoplasms, except for a dose-related sex- and strain-specific increase in mainly benign kidney tumours in mice.

Chloroform has also been tested in *in vitro* mutagenicity tests. It was not mutagenic in Chinese hamster V79 cells at the 8-azaguanine locus (Sturrock, 1977). Neither was it mutagenic in *Escherichia coli* K.12 or *Salmonella typhimurium* TA1535 or TA1538 (Greim, Bimboes, Egert, Goggelmann & Kramer, 1977), nor in TA1537, TA98 or TA100 (Simmon, Kauhanen & Tardiff, 1977), nor in TA1535, TA1537, TA1538, TA98 or TA100 (Daniel, Richold, Allen, Jones, Roe, Uttley & Van Abbé, 1980).

The purpose of the study reported in this paper was to extend the *in vitro* mutagenicity tests to include mutation in two other strains of *E. coli*, WP2p and WP2uvrA⁻p, using both plate incorporation and liquid pre-incubation, and to assess the damage done to human lymphocyte chromosomes and the induction of sister-chromatid exchanges by chloroform in the presence of rat-liver microsomes (S-9 mix).

Experimental

Reversion in E. coli. Two strains of *E. coli*, WP2p and WP2uvrA⁻p, kindly supplied by Dr S. Venitt of the Institute of Cancer Research, London, were used. Both are tryptophan auxotrophs and both carry the pKM101 Ampicillin resistance plasmid (McCann, Spingarn, Kobori & Ames, 1975). WP2p is fully DNA-repair proficient whereas WP2uvrA⁻p is deficient in excision repair at the uvrA locus. Both strains are reverted to prototrophy either by base change at the site of original alteration or by base change elsewhere in the genome so that the original defect is suppressed.

These two strains were treated with chloroform (Analar grade, BDH Chemicals Ltd, Poole, Dorset) in plate incorporation and pre-incubation tests both with and without rat-liver microsomes (S-9) prepared from Aroclor 1254-induced PVG/01a Hooded rats. In each case chloroform was administered at 10,000, 1000, 100, 10, 1 or 0·1 μg/plate using acetone as the diluent.

In the plate incorporation tests, the method was essentially that of Ames, McCann & Yamasaki (1975) for *S. typhimurium* in that to 2 ml of molten top agar (0·6% Lab M agar containing 0·5% NaCl, 5% stan-

dard nutrient broth and 5 μg L-tryptophan/ml) at 45°C were added 0·1 ml of a dilution of chloroform plus 0·1 ml (approximately 10^8 organisms) of an overnight broth culture of the tester bacteria, and, where appropriate, 0·5 ml of S-9 mix containing 10% S-9 with standard co-factors (Ames *et al.* 1975). These ingredients were rapidly mixed on a Whirlimixer and poured onto prepared Vogel–Bonner agar plates (Vogel & Bonner, 1956). Each treatment was carried out in triplicate, and the three plates for each dose either with or without S-9 were separately packed in gas-tight containers to avoid leakage of the volatile substances. The plates were incubated at 37°C for 48 hr, after which revertant colonies were counted on a Biotran II automatic colony counter (New Brunswick Scientific, Edison, NJ, USA).

In the pre-incubation tests, 0·5 ml of a dilution of chloroform was mixed with .0·5 ml of an overnight bacterial culture and, where appropriate, 2·5 ml of 10% S-9 mix. These mixtures were shaken at 200 oscillations/min for 20 min at room temperature on a Braun orbital shaker, and aliquots were added to top agar: 0·2 ml were taken from non-S-9 tubes and 0·7 ml from S-9 tubes, in order to give the same final volumes and concentrations as in the plate incorporation test. The top agar mixtures were poured on to Vogel–Bonner plates (three per treatment), incubated at 37°C for 48 hr and then the revertants were counted.

Negative controls were included in each experiment by replacing the chloroform with acetone, and positive controls were also included; *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG; Sigma London Chemical Co., Poole Dorset) was used at 1 or 10 μg/plate as a positive control without S-9 mix, and 2-aminoanthracene (2AA; Sigma Chemical Co.) was used at 5 or 50 μg/plate with 10% S-9 mix as a control chemical requiring metabolic conversion. Both plate incorporation and pre-incubation tests were repeated on separate days with fresh cultures, solutions and controls.

Chromosome breakage in human lymphocytes. Peripheral blood from a young, healthy volunteer with no history of chromosome fragility, no recent X-ray exposure and no recent virus infection was cultured in Hepes-buffered RMPI 1640 medium with glutamine, 20% foetal calf serum, and 100 units/ml each of penicillin and streptomycin (Gibco Europe Ltd, Paisley, Renfrewshire). Cultures (10 ml) were established in Sterilin disposable universal bottles and contained 0·4 ml blood, 0·1 ml phytohaemagglutinin (PHA; The Wellcome Foundation Ltd, Beckenham, Kent) and 0·1 ml preservative-free lithium heparin (Sigma Chemical Co.).

The cultures were incubated at 37°C for 24 hr, duplicate cultures were then treated with 0·1-ml aliquots of chloroform in acetone to give final concentrations of 50, 100, 200 and 400 μg/ml and 0·1 ml 10% S-9 mix was added to each culture. The treated cultures were incubated for 2 hr at 37°C. This method of treatment is a slight modification of that described by Thomson & Evans (1979). At the end of the treatment the cells were centrifuged at 800 rpm for 10 min, resuspended in fresh medium without PHA, and re-incubated for a further 22 hr to bring first cycle cells to mitosis after a total of 48 hr culture. One hour before harvest, colcemid (Gibco Europe Ltd) was added to each culture at a final concentration of 0·5 μg/ml. Cells were collected and fixed, and slides prepared, stained with Giemsa and mounted using conventional methods.

Negative controls were cultures treated with 0·1 ml acetone and 0·1 ml S-9 mix. Positive controls were not included since the donor's lymphocytes had previously shown dose-related chromosome breakage after treatment with benzo[*a*]pyrene in the presence of S-9 mix.

Slides were coded by an independent observer, and 100 well-spread metaphases with 46 or more chromosomes were scored from each culture, making a total of 200 cells per treatment. Abnormalities were classified as previously reported (Kirkland, Lawler & Venitt, 1978), and, using the system of Bauchinger, Schmid, Einbrodt & Dresp (1976), each abnormality was expressed in terms of the theoretical number of breaks necessary to produce a given lesion: a chromatid break, a chromosome break, or an acentric fragment were designated as one break, a dicentric or a rearrangement was designated as two breaks, *except* where a dicentric and acentric occurred in the same cell, when the total number of breaks equalled two and not three.

Sister-chromatid exchanges (SCE) in human lymphocytes. Cultures of peripheral blood from the same donor were established as above, but contained 50 μM-bromodeoxyuridine (BUdR) and were kept in the dark to avoid photolysis of BUdR-substituted DNA. Treatments were again for 2 hr in the presence of S-9 mix, but were begun 48 hr after establishment of the cultures, fresh BUdR medium without PHA being used to replace the treatment medium for the final 22 hr. The levels of chloroform used were 25, 50, 75, 100, 200 and 400 μg/ml final concentration. The negative control cultures contained acetone, and positive controls were not included since the donor's lymphocytes had previously shown a dose-related increase in SCE after treatment with benzo[*a*]pyrene in the presence of S-9 mix.

Accumulation of metaphase spreads, harvesting of cells and preparation of slides were as above. The dried slides were stored in the dark for at least 7 days after which the chromatids were differentiated by the method of Perry & Wolff (1974). The slides were again coded by an independent observer and, where possible, 50 well-differentiated metaphases with 46 or more chromosomes were scored from each culture, making a possible total of 100 cells per treatment.

Results

Reversion in E. coli

The results of the plate incorporation tests are shown in Table 1. It is clear that with chloroform all counts fall in the usual range expected for control counts. An analysis of variance on these figures confirmed that there was no dose-response. The bacteria and S-9 were not the cause of this lack of response since the positive control chemicals gave satisfactory reversion rates even though there was wide variation between the two experiments.

The results of the pre-incubation tests are shown in Table 2. With chloroform this method of treatment was more toxic than the plate incorporation method,

Table 1. *Plate incorporation assay of chloroform and positive control chemicals using* Escherichia coli *strains WP2p and WP2uvrA⁻p*

Chemical	Amount (μg/plate)	Mean number of revertants/plate in strain			
		WP2p		WP2uvrA⁻p	
		Without S-9 mix	With 10% S-9 mix	Without S-9 mix	With 10% S-9 mix
		Experiment 1			
Chloroform	0	8·7	5·0	5·3	4·0
	0·1	7·7	7·0	6·3	1·7
	1	11·0	4·7	6·3	3·7
	10	11·3	8·7	5·3	3·0
	100	8·3	3·7	7·0	8·3
	1000	5·0	4·0	16·7	4·0
	10,000	9·0*	4·7*	6·3*	3·3*
MNNG	0	6·7	—	6·7	—
	1	57·3	—	58·5	—
	10	498·3	—	644·0	—
2AA	0	—	8·0	—	22·5
	5	—	15·3	—	289·0
	50	—	18·5	—	386·0
		Experiment 2			
Chloroform	0	11·7	15·0	4·3	11·3
	0·1	8·3	8·3	9·7	20·3
	1	8·0	17·7	3·0	25·3
	10	9·3	14·7	6·7	17·3
	100	10·3	17·7	7·3	15·7
	1000	6·0	21·3	6·0	13·0
	10,000	16·0*	13·5*	4·0	14·0*
MNNG	0	12·0	—	14·7	—
	1	230·0	—	202·3	—
	10	1873·7	—	1091·7	—
2AA	0	—	27·7	—	51·7
	5	—	21·3	—	268·7
	50	—	34·7	—	752·3

MNNG = *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine 2AA = 2-Aminoanthracene
*The background lawn was sparse or absent on some or all of the plates.
Values are means for three plates.

and the control counts are generally higher, but there is no evidence of mutagenic activity. The positive controls were again satisfactory, but although there is variation between the experiments, both strains show better responses than the controls for the plate incorporation assay.

Chromosome breakage in human lymphocytes

The response of the donor's lymphocytes to chromosome breakage by benzo[*a*]pyrene treatment, with and without addition of S-9 mix, is shown in Fig. 1, in which all abnormalities have been calculated as theoretical lesions. It is clear that there is a dose-related increase in breakage with benzo[*a*]pyrene treatments, and this is confirmed by calculating correlation coefficients, which are 0·962 (without S-9) and 0·891 (with S-9).

The response of the same donor's lymphocytes to chloroform indicated random variation around the control value (Fig. 2). The highest breakage level was at 200 μg chloroform/ml with 8 breaks/100 cells compared with 5·5 breaks/100 cells in the control. However, comparing these levels by using the chi-square test with Yates correction shows that this difference is not significant ($\chi^2 = 0{\cdot}635$, $P > 0{\cdot}25$). The random nature of the breakage around control levels is confirmed by a correlation coefficient of 0·248.

SCE in human lymphocytes

The response of the donor's lymphocytes to SCE induction by benzo[*a*]pyrene is shown in Fig. 3. The control levels of SCE are quite high for this donor, being 16·375 SCE/cell without S-9 mix and 18·23 SCE/cell with the addition of S-9 mix. However, there is a significant increase in SCE with benzo[*a*]pyrene treatment and reasonable dose-response trends giving correlation coefficients of 0·782 (without S-9) and 0·995 (with S-9) over the range 0–10 μg/ml.

The response of the same donor's lymphocytes to chloroform is shown in Fig. 4. The control level of SCE (18·35 SCE/cell) closely resembles that seen in the benzo[*a*]pyrene control with S-9 mix, but at only two treatment levels are there more SCE/cell than in the control. At one of these (50 μg chloroform/ml) the SCE frequency is significantly different from the control ($\chi^2 = 11{\cdot}78$, $P < 0{\cdot}05$ with five degrees of freedom), but there is no dose-response trend, and at some doses (e.g. 25 μg/ml) the SCE frequency is significantly lowered ($\chi^2 = 26{\cdot}13$, $P < 0{\cdot}001$ with five degrees of freedom). This lack of dose-response is con-

Table 2. *Pre-incubation assay of chloroform and positive control chemicals using* Escherichia coli *strains WP2p and WP2uvrA⁻p*

Chemical	Amount (μg/plate)	Mean no. of revertants/plate in strain			
		WP2p		WP2uvrA⁻p	
		Without S-9 mix	With 10% S-9 mix	Without S-9 mix	With 10% S-9 mix
		Experiment 1			
Chloroform	0	24·0	17·7	33·3	40·0
	0·1	25·7	18·3	26·7	40·3
	1	17·7	17·7	2·3*	40·5
	10	22·7	18·3	NC*	42·3
	100	NC*	16·0	NC*	44·0
	1000	NC*	19·7	NC*	52·7
	10.000	NC*	NC*	NC*	NC*
MNNG	0	21·0	—	58·3	—
	1	878·0	—	455·7	—
	10	1403·3	—	1551·0	—
2AA	0	—	26·3	—	15·3
	5	—	52·0	—	152·3
	50	—	60·3		1239·3
		Experiment 2			
Chloroform	0	16·7	46·0	45·0	77·3
	0·1	21·3	32·3	48·7	60·0
	1	19·3	19·7	47·3	113·7
	10	22·0	27·7	53·0	56·3
	100	24·0	30·3	53·0	51·7
	1000	NC*	15·3*	NC*	10·7*
	10.000	NC*	NC*	NC*	NC*
MNNG	0	18·3	—	84·0	—
	1	482·0	—	463·0	—
	10	2209·7	—	1672·7	—
2AA	0	—	35·7	—	139·3
	5	—	81·7	—	1360·7
	50	—	102·3	—	1990·0

NC = Not counted MNNG = *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine
2AA = 2-Aminoanthracene

*The background lawn was sparse or absent on some or all of the plates.
Values are means for three plates.

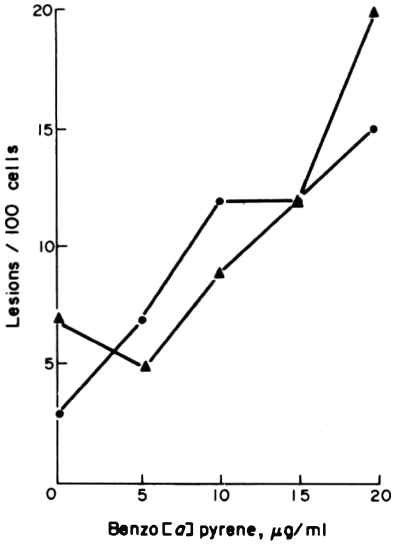

Fig. 1. Dose-related induction of chromosome breakage in human lymphocytes after treatment with benzo[*a*]pyrene with (▲) and without (●) metabolic activation (addition of S-9 mix). Abnormalities are expressed as the theoretical number of lesions necessary to produce the abnormalities according to the method of Bauchinger *et al.* (1976).

firmed by the correlation coefficient which is −0·206 for the whole dose range and is only 0·596 for the range 0–75 μg/ml.

Discussion

In this study chloroform has been shown not to be mutagenic in three *in vitro* tests with different genetic endpoints. Since the start of this work, we have learned that chloroform was one of the substances

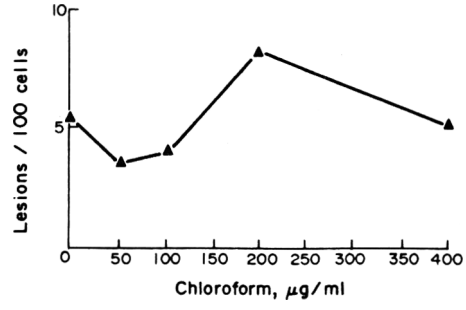

Fig. 2. Lack of effect of treatment with chloroform plus S-9 mix on chromosome breakage in human lymphocytes. obtained from the same donor as for Fig. 1.

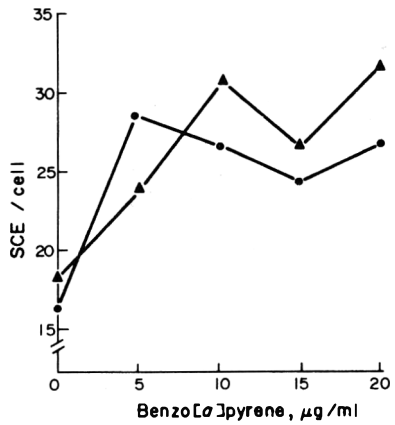

Fig. 3. Significant and dose-related induction of sister-chromatid exchanges (SCE) in human lymphocytes after treatment with benzo[*a*]pyrene with (▲) and without (●) S-9 mix.

assessed in the international programme for the evaluation of short-term tests for carcinogens (de Serres & Ashby, 1981), and out of 40 assays, 33 were negative, and seven gave positive results which were unreproducible, giving negative results on repeat. These tests included bacterial tests with Salmonella and *E. coli*, DNA repair in bacteria, mammalian cell transformation, unscheduled DNA synthesis in HeLa cells, the dominant lethal test, the micronucleus test and SCE induction in CHO cells.

The results reported here confirm the non-mutagenicity of chloroform in *E. coli*, even after pre-incubation, and extend the negative cytogenetic observations to breakage and SCE induction in human lymphocytes. This total failure of chloroform to cause any type of mutagenic event *in vitro* raises serious questions in relation to the prediction of cancer risk, particularly because in three animal studies (Eschenbrenner & Miller, 1945; National Cancer Institute, 1976; Roe *et al.* 1979) signs of carcinogenicity were observed when the dose levels greatly exceeded normal exposure levels in human use. However, in two of these studies, liver toxicity was observed and it may be that chloroform only acts as a carcinogen when it causes sufficient tissue damage to simulate partial hepatectomy. The development of kidney tumours under certain experimental conditions may likewise be directly related (Moore, Chasseaud, Majeed, Prentice, Roe & Van Abbé, 1980) to the nephrotoxic action of chloroform at high doses. This may explain why with lower doses of chloroform that are insufficient to cause liver or kidney regeneration, there was no increase in tumours in the rats, mice and dogs treated with toothpaste (Heywood *et al.* 1979; Palmer *et al.* 1979; Roe *et al.* 1979).

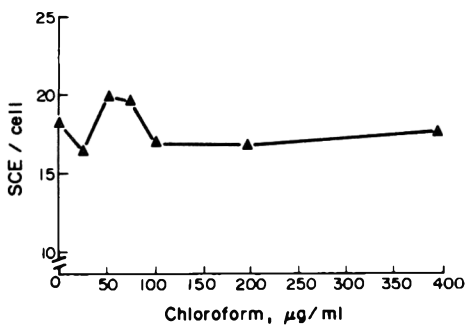

Fig. 4. Lack of effect of chloroform plus S-9 mix on the induction of sister-chromatid exchanges (SCE) in human lymphocytes obtained from the same donor as for Fig. 3.

An additional feature of the work reported here is that this appears to be the first experimental investigation of the response of human cells to chloroform. The view given in the IARC monograph on chloroform (IARC, 1979) that the compound should, for practical purposes, be regarded as if it presented a carcinogenic risk to humans, is not supported by the results of the studies reported here.

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SAFROLE: ITS METABOLISM, CARCINOGENICITY AND INTERACTIONS WITH CYTOCHROME *P*-450

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Summary—A review of studies on safrole metabolism shows that the compound gives rise to a large number of metabolites by two major pathways, oxidation of the allyl side chain and oxidation of the methylenedioxy group with subsequent cleavage to form a catechol. The mechanism by which safrole exerts the weak hepatocarcinogenicity that has been demonstrated in rats and mice is considered on the basis of published work and recent studies by the authors. Metabolic conversion of the allyl group gives rise to intermediates capable of covalent binding with DNA and protein, and recent findings are compatible with conversion of the methylenedioxy group to a carbene, which forms ligand complexes with the haem moiety of cytochromes *P*-450 and *P*-448. It is suggested that while the allyl group is responsible for the mutagenic potential of safrole, the methylenedioxy moiety may be associated with epigenetic aspects of carcinogenicity.

Introduction

Safrole (4-allyl-1,2-methylenedioxybenzene) is a natural plant constituent, found in oil of sassafras and certain other essential oils (Arctander, 1960; Fishbein & Falk, 1969; Friedman & Shibko, 1969; Guenther, 1948–1952). It is a member of the methylenedioxybenzene group of compounds, many of which (e.g. piperonyl butoxide) are extensively used as insecticide synergists.

A major source of human exposure to safrole is through consumption of spices, such as nutmeg, cinnamon and black pepper, in which safrole is a constituent (*Fenaroli's Handbook of Flavor Ingredients*, 1971; Friedman & Shibko, 1969; Synerholm & Hartzell, 1945; Weil, 1965). Safrole is also present in root beer, and has been used as an additive in chewing gum, toothpaste, soaps and certain pharmaceutical preparations (Fishbein & Falk, 1969).

Safrole is a weak hepatocarcinogen (Homburger, Kelley, Friedler & Russfield, 1961; Long, Nelson, Fitzhugh & Hansen, 1963) and it is a matter of considerable interest whether the allyl moiety or the methylenedioxy group, or both, are involved in the mechanism of its carcinogenesis.

Metabolism

The metabolism of safrole both *in vivo* and *in vitro*, using hepatic homogenates and cell cultures, has been the subject of many studies (Borchert, Wislocki, Miller & Miller, 1973b; Janiaud, Delaforge, Levi, Maume & Padieu, 1977; Stillwell, Carman, Bell & Horning, 1974), and a single study dealing with its metabolism in humans has also been reported (Benedetti, Malnoë & Broillet, 1977). Safrole is extensively metabolized, giving rise to a large number of metabolites. Metabolism involves essentially two major routes, oxidation of the allyl side chain, and oxidation of the methylenedioxy group with subsequent cleavage to form the catechol (Fig. 1).

Oxidation of the allyl chain

Safrole undergoes oxidation of the allylic group to yield the 2′,3′-epoxide (safrole epoxide) in both rat and guinea-pig (Janiaud *et al.* 1977; Stillwell *et al.* 1974). This epoxide is only a minor metabolite, possibly because of its slow rate of formation, or because of its further metabolism by epoxide hydratase to the corresponding dihydrodiol (Delaforge, Janiaud, Chessebeuf, Padieu & Maume, 1976; Delaforge, Janiaud, Levi & Morizot, 1980c). The dihydrodiol is one of the metabolites of safrole in rats and guinea-pigs, and presumably arises from the hydration of the 2′,3′-epoxide, as administration of the epoxide to these species resulted in excretion of the dihydrodiol in the urine (Borchert *et al.* 1973b; Delaforge *et al.* 1980c; Stillwell *et al.* 1974); the unchanged epoxide was also excreted in the urine, demonstrating its relative stability. Furthermore, the epoxide was detected in the liver microsomes of rats pretreated with safrole and in rat hepatocytes incubated with safrole (Delaforge, Janiaud, Maume & Padieu, 1978).

It is believed that the carcinogenicity of safrole is at least partly mediated through its metabolite, 1′-hydroxysafrole. This metabolite has been detected in the liver, urine and bile of animals, and is also found in the urine conjugated with glucuronic acid (Borchert, Miller, Miller & Shires, 1973a; Borchert *et al.* 1973b; Janiaud *et al.* 1977; Stillwell *et al.* 1974). The 1′-hydroxysafrole forms an ester, when incubated with cytosolic fractions from mouse and rat liver in the presence of 3′-phosphoadenosine-5′-phosphosulphate (Wislocki, Borchert, Miller & Miller, 1976). These workers also reported that alkaline digestion of hepatic protein, isolated from animals treated with 1′-hydroxysafrole, released a metabolite which appeared to be 3′-methylmercaptoisosafrole, indicating the further reaction of the hydroxylated metabolite with tissue *S*-proteins or with glutathione. 1′-Hydroxysafrole, like its parent compound, safrole, undergoes oxidation of the allyl group to yield 1′-hydroxy-2′,3′-epoxide (Wis-

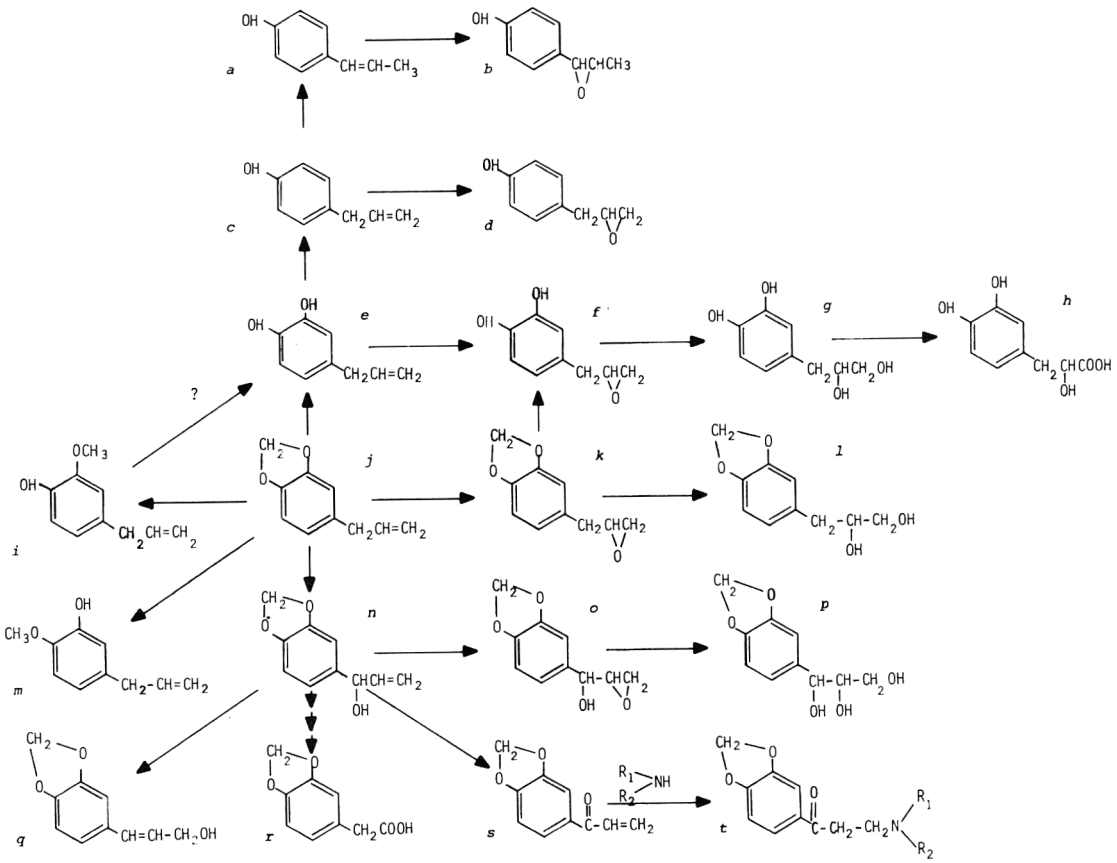

Fig. 1. Major routes of safrole metabolism: (a) propen-1′-ylphenol; (b) 1′,2′-epoxypropylphenol; (c) allylphenol; (d) 2′,3′-epoxypropylphenol; (e) allylcatechol; (f) 2′,3′-epoxypropylcatechol; (g) 2′,3′-dihydroxypropylcatechol; (h) 2′-hydroxy-3′-(3,4-dihydroxyphenyl)propanoic acid; (i) eugenol—(4-allyl-2-methoxyphenol); (j) safrole—(4-allyl-1,2-methylenedioxybenzene); (k) safrole epoxide; (l) dihydroxysafrole; (m) 1-methoxy-2-hydroxy-4-allylbenzene; (n) 1′-hydroxysafrole; (o) 1′-hydroxy-2′,3′-epoxysafrole; (p) trihydroxysafrole; (q) 3′-hydroxysafrole; (r) 1,2-methylenedioxybenzene-4′-acetic acid; (s) 1′-oxosafrole; (t) 3′-dialkylamino-2′,3′-dihydro-1′-oxosafrole.

locki *et al.* 1976), and incubation of 1′-hydroxysafrole with mouse- and rat-liver microsomal preparations and NADPH generated the hydroxy-epoxide. However, free 1′-hydroxyepoxysafrole has not been detected *in vivo*, although the glucuronic acid conjugate has been detected by gas-liquid chromatography–mass spectrometry of its trimethylsilyl derivative in the urine of animals dosed with safrole (Levi, Janiaud, Delaforge, Morizot, Maume & Padieu, 1977). This hydroxy-epoxide also serves as a substrate of epoxide hydratase, being converted to the trihydroxysafrole (Delaforge *et al.* 1976; Stillwell *et al.* 1974).

Administration of safrole or 1′-hydroxysafrole to animals or man gave rise to the excretion of its isomer, 3′-hydroxysafrole (Benedetti *et al.* 1977; Janiaud *et al.* 1977; Peele & Oswald, 1978), which is believed to result from rearrangement of the 1′-hydroxysafrole during enzymic hydrolysis with β-glucuronidase (Borchert, Miller & Miller, 1971). Other metabolites of 1′-hydroxysafrole include 3,4-methylenedioxyphenyl vinyl ketone, also known as 1′-oxosafrole (Peele & Oswald, 1978), and the formation of this metabolite was also indicated by the excretion of small amounts of adducts of oxosafrole with secondary amines (McKinney, Oswald, Fishbein & Walker, 1972; Oswald, Fishbein & Corbett, 1969; Oswald, Fishbein, Corbett & Walker, 1971).

Oxidation of the methylenedioxy group

The principal route of metabolism of safrole is through cleavage of the methylenedioxy group, the major metabolites being allylcatechol and its isomer, propenylcatechol. Eugenol and its isomer 1-methoxy-2-hydroxy-4-allylbenzene have been detected as minor metabolites in the rat, mouse and man (Benedetti *et al.* 1977; Janiaud *et al.* 1977; Stillwell *et al.* 1974). The intact allyl side chain of allylcatechol may be oxidized to yield 2′,3′-epoxypropylcatechol, which serves as a substrate for epoxide hydratase and is hydrated to 2′,3′-dihydroxypropylcatechol; this in turn is oxidized to the corresponding propanoic acid (Delaforge *et al.* 1976; Stillwell *et al.* 1974). The epoxide of allylcatechol may also be generated from the cleavage of the methylenedioxy group of the safrole epoxide. Using sensitive techniques, such as high-resolution capillary columns, 1′,2′-epoxypropylphenol and its isomer 2′,3′-epoxypropylphenol, as well as 2′,3′-epoxypropylcatechol, have been detected as minor metabolites in rat urine (Delaforge *et al.* 1976). These epoxides are

poor substrates for epoxide hydratase. Finally the acids, methylene-3,4-dioxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid, have been detected in the urine and bile of animals treated with safrole.

The cleavage of the methylenedioxy ring and the metabolism of the allyl group involve the hepatic microsomal mixed-function oxidases (Casida, 1970; Hodgson & Philpot, 1974). Administration to rats of the typical inducers of the mixed-function oxidases, phenobarbital and 3-methylcholanthrene, resulted in increased urinary excretion of epoxypropylphenol, epoxysafrole and 1'-hydroxysafrole (Borchert *et al.* 1973b; Janiaud *et al.* 1977), and phenobarbital stimulated the production of other epoxy and hydroxy derivatives and the conjugation of metabolites with glucuronic acid (Janiaud *et al.* 1977).

Carcinogenicity of safrole

The carcinogenic properties of safrole have been the subject of several studies and the compound is described as a weak hepatocarcinogen in both mice and rats (Borchert *et al.* 1973a; Epstein, Fujii, Andrea & Mantel, 1970; Hagan, Jenner, Jones, Fitzhugh, Long, Brouwer & Webb, 1965; Homburger, Kelley, Baker & Russfield, 1962; Homburger *et al.* 1961; Long, Hansen & Nelson, 1961; Parke & Gray, 1978). When fed to rats for 2 yr, a diet containing less than 1000 ppm safrole caused minimal hepatic damage, malignant changes being evident only at higher doses (Hagan *et al.* 1965; Long *et al.* 1963). The isomer, isosafrole, and dihydrosafrole exhibited even weaker hepatocarcinogenicity (Hagan *et al.* 1965); however, the latter compound fed for long periods to rats at a dose of 5000 ppm in the diet produced benign and malignant oesophageal tumours. Short-term administration of high doses of all three compounds resulted in extensive gross pathological damage (Taylor, Jenner & Jones, 1964). Liver is not the only tissue damaged by safrole; lymphomas and adenomas of the lung have also been described (Taylor *et al.* 1964). Neither allylbenzene nor methylenedioxybenzene show any significant toxicity when compared with safrole (Hagan *et al.* 1965), indicating that both the methylenedioxybenzene group and the allyl side chain are concerned in the manifestation of safrole toxicity.

The monohydroxylated derivative of safrole, 1'-hydroxysafrole, is more hepatotoxic and hepatocarcinogenic to animals than is the parent compound when fed at the same dietary level, indicating that it may act as a proximate carcinogen of safrole (Borchert *et al.* 1973a,b; Wislocki *et al.* 1976; Wislocki, Miller, Miller, McCoy & Rosenkranz, 1977). When administered to rats, 1'-hydroxysafrole labelled with tritium gives rise to tritium-labelled DNA, RNA and protein, indicating the covalent binding of the compound or of a further metabolite. Furthermore, application of the electrophilic 1'-hydroxysafrole epoxide, derived from 1'-hydroxysafrole, to mouse skin followed by repeated applications of the tumour promoter, croton oil, resulted in the formation of skin papillomas (Wislocki *et al.* 1977). The reactivity of 1'-acetoxysafrole with nucleosides and with methionine led to speculation that this might also act as an ultimate carcinogen (Borchert *et al.* 1973b; Wislocki *et al.* 1976), and when injected into newborn mice this compound gave rise to liver tumours, similar to those induced by 1'-hydroxysafrole (Borchert *et al.* 1973a). However, this metabolite could not be detected following incubation of 1'-hydroxysafrole with microsomes or cytosol from rat or mouse liver in the presence of acetyl-CoA (Wislocki *et al.* 1976). Another possible ultimate carcinogen, 1'-oxosafrole, did not result in carcinogenicity when administered orally to rats (Wislocki *et al.* 1977).

The bacterial system devised by Ames (Ames, McCann & Yamasaki, 1975) has been used extensively in studies to establish the identity of the ultimate carcinogen(s) of safrole. With the standard test system, safrole does not give a positive mutagenic response (McCann, Choi, Yamasaki & Ames, 1975; Swanson, Chambliss, Blomquist, Miller & Miller, 1979; Wislocki *et al.* 1977). However, when safrole was pre-incubated with liver microsomes from 3-methylcholanthrene-treated animals, a positive mutagenic response was obtained (Dorange, Janiaud, Delaforge, Levi & Padieu, 1978), although these findings could not be reproduced by other workers (Swanson *et al.* 1979). Green & Savage (1978) obtained a positive mutagenic response for safrole in the Ames test using other bacterial strains and a mouse-liver microsomal preparation, and also in a host-mediated system *in vivo*.

1'-Hydroxysafrole is more mutagenic than safrole but is still a relatively weak mutagen. Its mutagenicity was increased in the presence of an activating system, indicating that it is metabolized, at least partly, to more potent mutagen(s) (Swanson *et al.* 1979). However, other workers reported no positive mutagenic response either in the presence or absence of an activation system (Dorange, Delaforge, Janiaud & Padieu, 1977; McCann *et al.* 1975; Wislocki *et al.* 1977). The low mutagenicities of safrole and its 1'-hydroxy derivative probably reflect the low rates of metabolism observed *in vitro* with hepatic microsomes (Wislocki *et al.* 1976). In contrast, all of the epoxides of safrole that were investigated were found to be mutagenic in the Ames test, in the absence of an activation system (Dorange *et al.* 1977; Swanson *et al.* 1979; Wislocki *et al.* 1977). 1'-Acetoxysafrole, a postulated ultimate carcinogen, was also directly mutagenic in the Ames test (McCann *et al.* 1975; Wislocki *et al.* 1977) but no significant mutagenic response was obtained from dihydrosafrole, 3'-hydroxyisosafrole, 3'-acetoxyisosafrole and 1'-oxosafrole, with or without activation (Wislocki *et al.* 1977).

Interactions of safrole with cytochrome *P*-450

Safrole, as a substrate of the hepatic microsomal mixed-function oxidases, interacts with cytochrome *P*-450, giving rise to a type I spectral change (Franklin, 1971). In the presence of either NADPH and oxygen, or cumene hydroperoxide, it is transformed into a reactive intermediate which interacts with the sixth ligand of ferri- and ferrocytochrome *P*-450 to yield characteristic complexes (Elcombe, Bridges, Gray, Nimmo-Smith & Netter, 1975; Franklin, 1971 & 1976; Kulkarni & Hodgson, 1978; Parke & Rahman, 1971). The complex formed with reduced cytochrome

P-450 exhibits absorption maxima at 427 and 455 nm, while only one absorption maximum at 437 nm is observed with the oxidized cytochrome (Elcombe *et al.* 1975; Gray & Parke, 1973; Hodgson & Philpot, 1974; Lake & Parke, 1972). Safrole does not form the complex when incubated with microsomes plus dithionite or NADH, indicating that the ligand is formed by a reactive intermediate of safrole and not by safrole itself (Elcombe *et al.* 1975). The formation of the complex *in vivo* has also been shown to occur in rats and mice following administration of safrole (Delaforge, Ioannides & Parke, 1980a; Fennell, Sweatman & Bridges, 1980; Parke & Rahman, 1971).

The nature of the reactive safrole intermediates remains unclear, but it is generally believed that the complex is formed between the cytochrome and a carbene generated from the hydroxylation of the methylenedioxy group with subsequent loss of water (Mansuy, Battioni, Chottard & Ullrich, 1979; Nastainczyk, Ullrich & Sies, 1978; Ullrich, Nastainczyk & Ruff, 1975). Carbenes are known to have a high affinity for reduced cytochrome *P*-450 (Mansuy, Nastainczyk & Ullrich, 1974). Other possible reactive intermediates for safrole have been suggested, such as carbanions formed by removal of a proton from the methylene group (Ullrich & Schnabel, 1973), radicals generated following the homolytic scission of the methylenedioxy group (Hansch, 1968), and benzodioxolium ions produced following the loss of a hydride ion from the methylenedioxy bridge (Hennessy, 1965).

Experimental evidence for the involvement of hydroxyl radicals in the formation of the ligand complex of safrole and cytochrome P-*450*

The formation of the reactive intermediate that gives rise to the formation of the safrole–cytochrome *P*-450 ligand complex may involve hydroxylation by cytochrome *P*-450 or interaction with hydroxyl or other radicals.

Recent work has demonstrated that NADPH-cytochrome *P*-450 reductase can generate hydroxyl radicals (Lai, Grover & Piette, 1979). To investigate the role of hydroxyl radicals in the formation of the safrole ligand complex, we incubated hepatic microsomes with NADPH and safrole in the presence of scavengers of the active forms of oxygen. In addition to the study of the effects on safrole, the microsomal oxidation of methional to ethylene, a reaction effected by hydroxyl radicals, was also investigated.

Male Wistar albino rats (150–200 g) received a daily intraperitoneal administration of phenobarbital (80 mg/kg) for 3 days and were killed 24 hr after the last administration. Hepatic microsomal preparations were prepared as previously described (Ioannides & Parke, 1975). To study the generation of the ligand complex *in vitro*, microsomal fractions (1 mg protein/ml) were incubated with safrole (0·04–0·5 mM) and various oxygen scavengers at concentrations shown in Table 1 (with the data obtained with 0·3 mM-safrole). Following addition of NADPH (0·5 mM), the formation of the ligand complex was monitored for 2 min by measuring the optical difference between 455 and 490 nm using a double-beam dual-wavelength recording spectrophotometer.

The antioxidants butylated hydroxytoluene (BHT) and ascorbic acid inhibited the formation of the safrole complex with cytochrome *P*-450, suggesting the requirement for oxidation in the formation of the complex. The hydroxyl-radical scavengers mannitol, dimethylsulphoxide and methional inhibited the formation of the ligand complex by 30% (Table 1). Lineweaver-Burk presentations, such as that for methional in Fig. 2, showed that with these four compounds inhibition was likely to be of the competitive type. In contrast, the inhibition by catalase and superoxide dismutase was uncompetitive, indicating that these do not compete directly with safrole for hydrogen peroxide. It is possible that catalase, by removing H_2O_2, prevents its further conversion to hydroxyl radicals in the presence of ferrous ions (Walling, Partch & Weil, 1975).

The involvement of the OH· radical in the formation of the ligand complex was further indicated by the decreased evolution of ethylene from methional in the presence of safrole. Aliquots of a liver-microsomal preparation from phenobarbital-treated rats (3 mg protein/ml 0·1 M-phosphate buffer, pH 7·4) were incubated with an NADPH-generating system (2 μmol NADP, 20 μmol glucose 6-phosphate and 2 units glucose 6-phosphate dehydrogenase) in rubber-sealed tubes and the reaction was started by addition of methional (0–0·4 mM). Ethylene evolution was determined by removing 1-ml aliquots and injecting them into a Packard 409 gas chromatograph equipped with

Table 1. *Effect of oxygen scavengers on the formation of the safrole complex with cytochrome* P-*450 in rats pretreated with phenobarbital*

Oxygen scavenger	Concentration	Complex formation (% of control)
Butylated hydroxytoluene	60 μm	55
Ascorbic acid	600 μm	68
Dimethylsulphoxide	10 mM	70
Mannitol	6 mM	71
Methional	600 μm	66
Catalase	3000 IU	72
Superoxide dismutase	500 IU	80

Incubations were carried out at a safrole concentration of 0·3 mM. Reactions were started by addition of NADPH (0·5 mM). Control incubations contained 0·3 mM safrole and no oxygen scavenger.

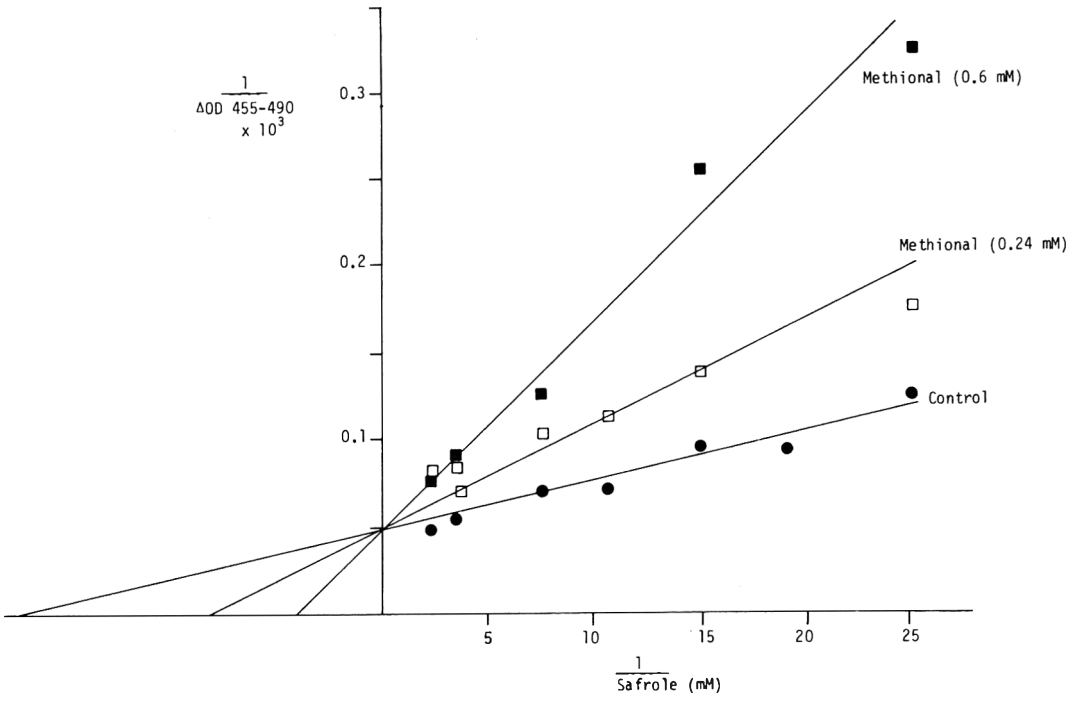

Fig. 2. Liver microsomes from phenobarbital-treated rats (1 mg protein/ml) were incubated with safrole (0·04–0·5 mM). The reaction was commenced by addition of NADPH (0·5 mM) and the initial velocity of the ligand complex formation was monitored for 2 min.

a 2 m × 2 mm glass column packed with Carbosieve B (60-80 mesh). Analysis was carried out isothermally at 170°C using a flame ionization detector and helium or oxygen-free nitrogen as carrier gas (25 ml/min); air and hydrogen flows were 300 and 30 ml/min respectively, and the chromatograph was operated with an injection temperature of 200°C and detection temperature of 250°C; under these conditions ethylene had a retention time of 2·2 min, and the response was linear from 0·5 pmol to at least 200 pmol/ml. Safrole at concentrations of 2 and 5 mM inhibited, possibly competitively, the release of ethylene from methional, confirming the involvement of the OH· radical in the metabolism of safrole (Fig. 3). This involvement of the OH· radical in the formation of a ligand complex between safrole and cytochrome *P*-450 is compatible with the formation of a carbene (Fig. 4).

Inhibition of mixed-function oxidase activity by safrole

The formation of the safrole carbene complex is accompanied by a decrease in the concentration of free cytochrome *P*-450 and by a concomitant loss of mixed-function oxidase activity (Anders, 1968; Franklin, 1972; Parke & Rahman, 1971). The characteristic ligand complex of reduced cytochrome *P*-450 and carbon monoxide, with the absorption maximum at 450 nm, is decreased, showing that carbon monoxide cannot displace the carbene from the sixth ligand of the haem iron. An inverse linear relationship ($r = 0{\cdot}96$) exists between the ratio of free and total cytochrome *P*-450 concentrations, and the ratio of carbene-bound and total cytochrome *P*-450 concentrations *in vivo* when safrole was administered to the rat (Delaforge *et al.* 1980a). A similar relationship was reported by the same workers for the formation of the complex *in vitro*.

In the presence of some type I and reverse type I substrates, but not of type II substrates (which ligand to the haem moiety), the ferricytochrome carbene complex dissociates, resulting in removal of the 455-nm peak and restoration of the catalytic activity of the cytochrome (Delaforge, Ioannides & Parke, 1980b; Dickins, Elcombe, Moloney, Netter & Bridges, 1979; Elcombe *et al.* 1975; Elcombe, Bridges & Nimmo-Smith, 1976; Gray & Parke, 1973; Ullrich, 1977), presumably because of removal of the haem-bound safrole metabolite. The extent of the increase in mixed-function oxidase following displacement is dependent on the amount of complex formed and the displacing conditions (Elcombe, Dickins, Sweatman & Bridges, 1977). Furthermore, when the substrates that act as displacers are incubated with microsomal preparations isolated from animals pretreated with safrole or isosafrole, only very weak binding spectra with oxidized cytochrome *P*-450 are exhibited, but these undergo a time-dependent intensification resulting from the displacement of the safrole complex (Gray & Parke, 1973). Displacement occurs anaerobically and does not involve competition for the type I binding site, since the carbene ligands to the haem iron and is not bound to the apoprotein. However, as no type II substrates act as displacers, the dissociation of the complex may be initiated by the breaking of some additional linkage of the carbene with the apoprotein, possibly involving the allyl side chain. Alternatively, the interaction of the displacing sub-

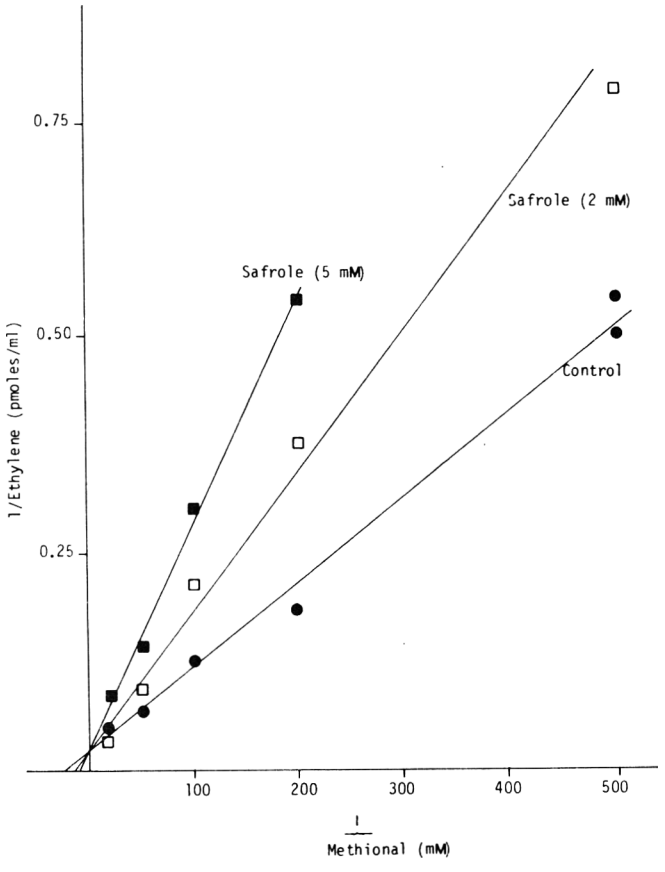

Fig. 3. Incubation mixtures comprised 10 ml liver microsomes from phenobarbital-treated rats (3 mg/ml), and methional (0·002–0·4 mM). The reaction was commenced by addition of an NADPH-generating system (2 μmol NADP, 20 μmol glucose 6-phosphate and 2 units glucose-6-phosphate dehydrogenase). Evolution of ethylene was determined by GLC as described.

strate with the type I site may lead to a conformational change of the cytochrome, resulting in the loss of the 'safrole' ligand and conversion of the cytochrome to the high-spin state.

In our studies (unpublished data, 1981), we have found that the carcinogen benzo[*a*]pyrene can also act as a displacer to remove the carbene and restore mixed-function oxidase activity. The ability of benzo[*a*]pyrene to displace the carbene, and then of the free cytochrome to convert the benzo[*a*]pyrene to mutagenic intermediates was investigated in safrole-treated rats. Mutagenicity was determined by the Ames test (Ames *et al.* 1975) using *Salmonella typhimurium* strain TA98. The activation system was prepared with microsomes from safrole-pretreated rats (single daily intraperitoneal injections of 150 mg/kg for 3 days, the animals being killed 24 hr after the last administration). Displacement of the carbene was achieved by pre-incubation of the microsomes with benzo[*a*]pyrene (2 μg) for 15 min prior to addition of the NADPH-generating system to initiate metabolism. Following pre-incubation, the number of his-

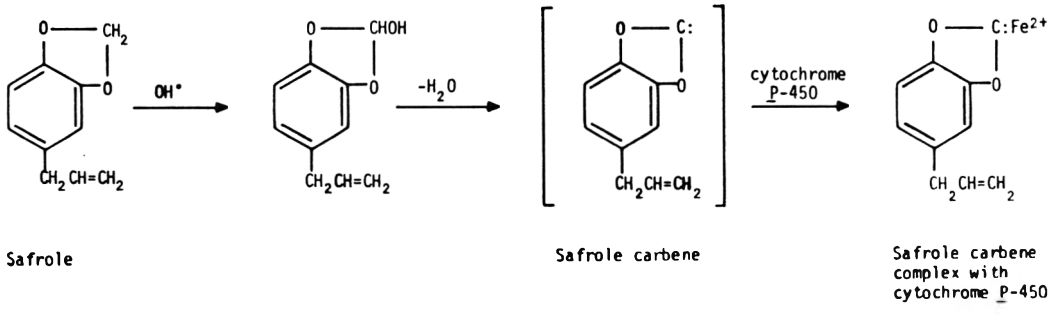

Fig. 4. Possible route of formation of a safrole carbene–cytochrome *P*-450 complex.

tidine revertants was more than doubled (from 128 to 288/plate, the number in the absence of any activation system being 47/plate), demonstrating that the function of the cytochrome was restored when the ligand complex was dissociated. When biphenyl, a non-mutagen, was used as the displacing agent and no benzo[*a*]pyrene was added, no mutagenic response was observed, demonstrating that the displaced carbene itself did not elicit the mutagenic response. Previous workers have failed to displace the safrole carbene with benzol[*a*]pyrene (Elcombe *et al.* 1977).

Dissociation of the safrole carbene ligand complex results spontaneously in the replacement of the 455 nm absorption maximum with a 450 nm peak, resembling the CO-difference spectrum with cytochrome *P*-450 (Delaforge & Coon, 1981; Hodgson & Philpot, 1974; Hodgson, Philpot, Baker & Mailman, 1973; Kulkarni & Hodgson, 1978). The presence of this CO was initially attributed to endogenous breakdown of haem (Schmid, 1973), but recent evidence suggests that it is generated from the methylenic carbon during the metabolism of methylenedioxyphenyl compounds by the cytochrome *P*-450 enzyme system (Yu, Wilkinson & Anders, 1980). Certain methylenedioxy compounds that generate CO do not elicit a 455 nm absorption maximum, showing that the carbene and CO are formed *via* different pathways, perhaps from a common unstable intermediate (Yu *et al.* 1980). The formation of CO subsequent to displacement of the safrole moiety from the ligand complex, indicates that the C atom of the methylenedioxy moiety is still intact, confirming the involvement of a carbene and indicating that the product of the displacement is likely to be an allylcatechol. The displaced product may then bind covalently to cytochrome *P*-450, inhibiting further formation of the ligand complex (Delaforge & Coon, 1981).

Ligand complex formation with cytochromes *P*-450 and *P*-448

Cytochrome *P*-450 exists in several forms, differing in their spectral, immunological and electrophoretic properties as well as in their structures and substrate specificity (Guengerich, 1979). One of these forms, namely, cytochrome *P*-448, predominates in foetal and neonatal rat liver and in malignant tissue, and is formed by the treatment of animals with hepatocarcinogens, including safrole and isosafrole (Parke, 1981). Cytochromes *P*-450 and *P*-448 form ligand complexes with safrole both *in vivo* and *in vitro*, showing that they can convert safrole to the carbene and then interact with it (Delaforge & Coon, 1981; Delaforge *et al.* 1980a). However, the safrole carbene complexes formed show different stability. The absorption maximum at 455 nm is formed less readily with cytochrome *P*-448, indicating that either the rate of generation of the carbene, or its interaction to form the complex, is slower with cytochrome *P*-448 than with cytochrome *P*-450. Furthermore, dissociation by biphenyl of the safrole complex formed *in vivo* in animals pretreated with the cytochrome *P*-450 inducer, phenobarbital, resulted in a large increase (150%) in the cytochrome *P*-450-mediated 4-hydroxylation of biphenyl but in only a small increase (20%) in the cytochrome *P*-448-mediated 2-hydroxylation of biphenyl (Delaforge *et al.* 1980b). In contrast, dissociation of the safrole complex formed in animals pretreated with the cytochrome *P*-448 inducer, 3-methylcholanthrene, resulted in a small increase (10%) in the 4-hydroxylation of biphenyl with no significant increase in the 2-hydroxylation, indicating that displacement of the ligand from the cytochrome *P*-448 form is less facile than displacement from the cytochrome *P*-450 complex. Inhibition of the mixed-function oxidases by piperonyl butoxide, another methylenedioxy compound, was more marked in animals pretreated with phenobarbital than in those treated with 3-methylcholanthrene, indicating that the piperonyl butoxide carbene also interacted more readily with cytochrome *P*-450 to form a complex (Anders, 1968; Franklin, 1972). Similar findings have been reported for SKF-525A and amphetamine ligand complexes (Buening & Franklin, 1974; Franklin, 1974).

The ligand complex formed with cytochrome *P*-450 is metastable, 50% being destroyed within 6 hr following safrole administration to rats (Delaforge *et al.* 1980a). In contrast, the complex with cytochrome *P*-448, although more slowly formed, is more stable, no degradation being evident in the first 6 hr. If indeed CO generation is directly related to the ligand complex formation (Yu *et al.* 1980) these findings may explain why rat-liver microsomes induced with phenobarbital, but not those induced with 3-methylcholanthrene, lead to CO production on dissociation of the safrole carbene complex.

A novel haemoprotein formed by safrole and isosafrole

Administration of safrole to animals leads initially to a marked inhibition of the mixed-function oxidases (Anders, 1968; Fujii, Jaffe, Bishop, Arnold, Mackintosh & Epstein, 1970; Hodgson & Casida, 1961; Nakatsugawa & Dahm, 1967). The inhibition is achieved through two different mechanisms, on the one hand competitive inhibition by safrole itself of the substrate for metabolism, and on the other formation of the safrole carbene ligand complex, preventing the ready hydroxylation of the substrate.

However, like many inhibitors, safrole and isosafrole also stimulate the synthesis of new enzymic protein and act as potent inducers of the mixed-function oxidase system (Lotlikar & Wasserman, 1972; Parke & Rahman, 1970). The pattern of induction has characteristics of both the barbiturate and the polycyclic aromatic hydrocarbon inducers (Fennell *et al.* 1980; Gray, Parke, Grasso & Crampton, 1972; Lake & Parke, 1972; Wagstaff & Short, 1971). Administration of a safrole-containing diet (0·25%) to rats resulted in induction of hepatic cytochrome *P*-450 and cytochrome *P*-448 activities within 7 days (Parke & Gray, 1978). Extrahepatic mixed-function oxidase activities, such as those of the small intestine and kidney, were also induced by isosafrole (Lake, Hopkins, Chakraborty, Bridges & Parke, 1973). The full extent of the inducibility of the mixed-function oxidases is only evident when the carbene ligand complex with cytochrome *P*-450 is dissociated to release the free cytochrome. In rats pretreated with safrole, there were

increases in both cytochrome *P*-450 and *P*-448 activities following dissociation of the ligand complexes with biphenyl (Delaforge *et al.* 1980b). These observations indicate that safrole induces a haemoprotein having overlapping activities of cytochrome *P*-450 and cytochrome *P*-448, or may induce a mixture of the two. Indeed, using SDS-disc gel electrophoresis, it has been demonstrated that isosafrole induces a novel haemoprotein, which is distinct from those induced by phenobarbital or by 3-methylcholanthrene but which may be similar to the minor protein band induced by 3-methylcholanthrene (Fennell, Dickins & Bridges, 1979). Further work has led to the isolation from isosafrole-pretreated rats, and the purification of a unique form of hepatic cytochrome *P*-450 existing in the form of an isosafrole metabolite complex (Ryan, Thomas & Levin, 1980). The cytochrome *P*-448 induced by 3-methylcholanthrene has also been shown to contain a bound moiety of the inducing agent (Schenkman, Greim, Zange & Remmer, 1969).

Conclusions

This ligand complexing and covalent binding of reactive intermediates with the haem and protein moieties, respectively, of cytochrome *P*-450, may be fundamental to the mechanism of carcinogenesis. The structural and functional properties of the cytochrome are substantially changed by this binding which, since ribosomes are attached to the endoplasmic reticulum at cytochrome *P*-450, may be associated with the loss of ribosomes known to occur following treatment with safrole or other carcinogens (Parke, 1981). Chemical carcinogenesis is known to be associated with increases in hepatic cytochrome *P*-448 and simultaneous loss of cytochrome *P*-450 activity (Parke, 1981) and could, through loss of ribosomes, result in impairment of glycoprotein synthesis and so contribute to the process of malignant transformation by epigenetic mechanisms (Parke, 1981). Hence, of the two functional groups of safrole, the allyl group has been shown to be associated with mutagenicity, and the methylenedioxy moiety to be associated with changes to cytochrome *P*-450 and possibly with epigenetic aspects of carcinogenicity.

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SULPHITE TOXICITY: A CRITICAL REVIEW OF *IN VITRO* AND *IN VIVO* DATA

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Summary—Data on sulphite chemistry and toxicity in *in vitro* systems are reviewed from the perspective of potential mammalian toxicity. The observed toxicity of ingested sulphite in mammals is also summarized and the conclusions reached are compared with the results of the *in vitro* experiments. Information on sulphite metabolism is included to reconcile the different conclusions that may be drawn from these two sets of data. Consideration of data from all sources facilitates the selection of the specific reactions of sulphite most likely to be of toxicological significance in mammals.

Introduction

Sulphur dioxide (SO_2), sulphurous acid (H_2SO_3) and salts of sulphite (SO_3^{--}) and bisulphite (HSO_3^-) exhibit antioxidant and antimicrobial properties which make them logical choices as preservatives for certain foods and beverages. These quadrivalent-sulphur (S^{IV}) substances exist in a pH-sensitive equilibrium as described by the following series of equations:

$$SO_2 + H_2O \rightleftharpoons H_2SO_3 \overset{-H^+}{\rightleftharpoons} HSO_3^- \overset{-H^+}{\rightleftharpoons} SO_3^{--}$$

Which of the above chemical species predominates depends upon the pH and the acid dissociation constants, the latter in turn being somewhat dependent on temperature and ionic strength. For example, at 25°C the pK_a of HSO_3^- is 6·25 at high ionic strength and 7·2 at low ionic strength (Shapiro, 1977). Under physiological conditions (considered to be pH 7·4 and 37°C) an essentially exclusive mixture of SO_3^{--} and HSO_3^- will result, with the former species predominating, no matter which of the S^{IV} species is initially introduced. For convenience and to avoid confusion, 'sulphite' will be used throughout this paper for referring to any of these readily interconvertible S^{IV} species. However, when specific reference is made to one species only, the chemical formula of that compound or ion (e.g. HSO_3^-) will be used.

Sulphite is used extensively in wine making as a selective inhibitor of yeasts and bacteria and is present in finished wines in concentrations up to approximately 6 mM, although part of this sulphite is in combined form. Foods and other beverages to which sulphite is often added include dehydrated fruits, vegetables and soups, as well as fruit juices and beer (Institute of Food Technologists and Committee on Public Information, 1976). The mean daily intake of sulphite from the diet has been estimated by various sources to be between 0·0014 and 0·14 mmol/kg body weight (in the United States), although the latter figure was reported as a probable over-estimation. Bigwood (1973) estimated that the mean daily intake of sulphite among adults in Belgium was in the range of 0·0011–0·016 mmol/kg. Because of the high concentration of sulphite in many wines and the wide personal variation in wine consumption, it follows that individual consumption of sulphite also varies considerably. Therefore, there is probably a small percentage of the population that takes in amounts of sulphite greatly in excess of these estimated averages.

Sulphite is also taken into the body during the inhalation of air polluted with SO_2. Although the focus of this article is on ingested sulphite, inhaled SO_2 cannot be totally dissociated from this source since it also adds to the body burden of 'exogenous' sulphite, albeit usually to a minor degree. One can calculate, for example, that the daily intake of sulphite due to inhalation of atmospheric SO_2 at the maximum 24-hr average permitted by the EPA (0·14 ppm) would be approximately 25 times less than the sulphite taken in by drinking 250 ml of wine containing sulphite at a level of 5 mM. However, although the subject is outside the scope of this article, it is important to emphasize here that the localized effects of SO_2 on the pulmonary system may be of much greater significance than the absolute amount of SO_2 absorbed.

The subject material of this paper is organized into several areas. The first of these consists of *in vitro* reactions of sulphite with biological compounds under approximately physiological conditions. These data are considered from the perspective of their implications for mammalian toxicity. Next, the *in vitro* modification of enzyme activity by sulphite and the toxicity of sulphite in biologically active *in vitro* assay systems are considered, followed by a review of toxicological data gathered from *in vivo* experiments in mammals. The metabolism of sulphite in mammals is summarized and finally some general observations and conclusions are presented. The literature references, although intended to be sufficiently extensive to be representative, are not necessarily comprehensive. Greater detail in some subject areas can be obtained from two excellent reviews, one by Shapiro (1977) and the other by Hayatsu (1976).

Reactions of sulphite *in vitro*

Addition to aldehydes and ketones

Sulphite reacts reversibly with open-chain aldehydes and ketones to form hydroxysulphonate compounds (Petering & Shih, 1975; Schroeter, 1966). The stability of these adducts varies considerably depending upon the pH and especially upon the reactive species. For example, at pH 7 the apparent dissociation constant (K_d) of glucose hydroxysulphonate is approximately 2·2 M (Vas, 1949), while that of the formaldehyde adduct is 8×10^{-5} M (Dasgupta, DeCesare & Ullrey, 1980). Vas (1949) determined that the velocity constant for the decomposition of glucose hydroxysulphonate at pH 6·1 and 20°C is approximately 1/min (the value at pH 7 was not determined). Extrapolation of these constants to the *in vivo* situation indicates that sulphite would be required to be present continuously if even a small amount of glucose were to be maintained as the hydroxysulphonate adduct.

Other physiological aldehydes and ketones, such as pyruvate, α-ketoglutarate and acetaldehyde, form hydroxysulphonate adducts that are considerably more stable than the glucose adduct. Burroughs & Sparks (1973) give the dissociation constants for pyruvate and acetaldehyde hydroxysulphonates at pH 7 and 20°C as $4·6 \times 10^{-4}$ M and $2·8 \times 10^{-6}$ M, respectively.

Ionic addition to C–C double bonds

The sulphite ion adds to some C–C double bonds forming sulphonic acid compounds. Under suitable conditions this reaction has been shown to occur with several molecules of extreme biological importance.

Addition to pyridine and flavin nucleotides. Sulphite adds reversibly to the 3–4 double bond of the pyridine ring of nicotinamide adenine dinucleotide (NAD), forming a sulphonate group at the active site for reduction by H^-. The stability of this adduct alone at pH 7·5 (25°C) is only moderate, its dissociation constant being approximately 3×10^{-2} M (Shih & Petering, 1973). However, when NAD^+ is associated with certain enzymes, its sulphite adduct is much more stable.

An analogous sulphite adduct is formed with flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) at the N_5 atom of the isoalloxazine ring, the usual site of reduction of the ring by H^-. These adducts are even less stable than the NAD–sulphite adduct (dissociation constants approximately 2 M), but as with the latter, their stability can be extensively enhanced when they are bound to protein (Müller & Massay, 1969). Since the sulphite adducts of NAD^+ and flavin coenzymes cannot accept H^- from the substrate, they cannot function in their usual capacity. Examples of this inhibition of enzyme function will be discussed later.

Addition to menadione. Vitamin K_3 (menadione) is a water-soluble synthetic form of vitamin K. Sulphite, at pH 7·4, adds to the 2–3 double bond of the naphthoquinone ring of menadione to form a sulphonate adduct. Since the natural forms of vitamin K (K_1 and K_2) are fat soluble, the reaction rates of menadione are not necessarily applicable to them. As with other reactions of this type, the addition of sulphite to menadione is reversible. The dissociation constant for the adduct is 10^{-6} M (Shih & Petering, 1973), indicating considerable stability of the adduct in the presence of free sulphite. However, the sulphite adduct is a source of vitamin K when fed to animals (Nir, Kafri & Cohen, 1978), suggesting that the adduct readily dissociates in the body.

Addition to uracil and cytosine. Sulphite adds reversibly to the 5–6 double bonds of uracil, uridine or uridine 5′-phosphate forming the 5,6-dihydro-6-sulphonate adduct (Hayatsu, 1976). The rate of the forward reaction (formation of the adduct) is most rapid at a pH of approximately 7 and the equilibrium shifts in the direction of the reverse reaction above and below that pH. The dependency of the reaction rate on sulphite concentration at pH 7 has been demonstrated in several studies (Hayatsu, Wataya, Kai & Iida, 1970; Pitman & Jain, 1979; Shapiro, Welcher, Nelson & Di Fate, 1976). According to Hayatsu *et al.* (1970), 86% of the uridine reactant at 22°C in 1 M-sulphite (excess) was in the form of the adduct after 0·5 hr, while in 0·1 M-sulphite only 12% of the uridine had reacted in the same period of time. At this pH, the equilibrium of the reaction was decisively in the direction of the adduct and in 1 M-sulphite essentially all of the uridine had been converted to the sulphonate after 1 hr. However, uridine can be regenerated from its sulphonate adduct at pH 7 by removal of free sulphite. The regeneration process (i.e. reversal of adduct formation) approximates first-order kinetics. At physiological pH and in the absence of sulphite, the half-life of 5,6-dihydrouracil-6-sulphonate is several hours. Regeneration occurs more rapidly as the pH rises and at or above pH 11 it is extremely rapid, the half-life of the sulphonate compound being a few seconds (Rork & Pitman, 1974).

Sulphonate adducts of cytosine and its derivatives, analogous to those of uracil, are formed during incubation with sulphite under the appropriate conditions. The extent of adduct formation is determined primarily by pH and the concentration of sulphite. The cytidine adduct is stable at acid pH, and in high concentrations of sulphite (about 0·5 M) approximately 80% of cytidine is in this form at equilibrium (Shapiro, Di Fate & Welcher, 1974). Compared to uridine adducts, cytidine adducts are relatively unstable at physiological pH, only 10% of the cytidine existing in the combined form at equilibrium under conditions of excess sulphite. Therefore, a high concentration of sulphite is required to maintain the presence of dihydrocytosine 6-sulphonate at physiological pH. Recent data suggest, however, that this adduct may be considerably more stable *in situ* in bacteriophage DNA (Sklyadneva, Chekanovskaya, Nikolaeva & Tikchonenko, 1979a). These data will be discussed later.

Deamination of 5,6-dihydrocytosine-6-sulphonate

Under the appropriate conditions, 5,6-dihydrocytosine-6-sulphonate can be deaminated to the corresponding uracil adduct. This deamination is catalysed by basic substances (including sulphite) and occurs at an optimal rate at pH 5 (Shapiro *et al.* 1974). Thus, *via* the deamination reaction and the addition reactions described above, cytosine can be converted to uracil with obvious toxicological implications. The

optimal pH for the overall conversion is 5, and at physiological pH the rate of conversion declines to approximately 1% of that observed at optimal pH. Further, at low sulphite concentrations compatible with the *in vivo* situation, the rate of conversion can be expected to be extremely low. Slae & Shapiro (1978) have estimated this rate to be $4{\cdot}0 \times 10^{-14}$/sec for a sulphite concentration of 10^{-3} mM.

Transamination of cytosine

Sulphite is capable of catalysing the transamination of cytosine and its derivatives with primary and secondary amines to produce N^4-substituted cytosines. The reactive intermediate for transamination is the 5,6-dihydrocytosine-6-sulphonate adduct discussed previously. Shapiro & Gazit (1977) have carried out transamination reactions at physiological temperature and pH between polylysine and cytidine, polycytidylic acid and lysine, and polylysine and polycytidylic acid. High concentrations of sulphite were used (approximately 1 M) and the reactions were allowed to proceed for hours or days. Crosslinking between monomers and polymers of lysine and cytosine was observed. The significance of these reactions lies in their logical extension to the crosslinking of nucleic acids and proteins. Although attempts to crosslink double-stranded native DNA with polylysine were not successful, evidence for crosslinking was found in a similar experiment involving DNA and histones (Shapiro & Gazit, 1977). Sklyadneva, Shie & Tikchonenko (1979b) were successful in crosslinking lysine with DNA isolated from S_d bacteriophage; 40% of the cytosine residues were transaminated during a 24-hr reaction in the dark at pH 6·25, 0·25 M-sulphite and 25°C.

Sulphitolysis reactions

Sulphitolysis of thiamine. The cleavage of thiamine by sulphite was described by Williams, Waterman, Keresztesy & Buchman (1935). This reaction is irreversible and involves a nucleophilic attack by sulphite on the quaternary nitrogen of the thiazole ring to yield pyrimidine sulphonic acid and 4-methyl-5-β-hydroxyethylthiazole. The kinetics of this reaction have been studied in detail by Leichter (1969) who showed that thiamine sulphitolysis is first order with respect to each reactant. At pH 5 and 25°C, the half-life of 10 μM-thiamine in the presence of 1·0 mM-sulphite is approximately 13 hr. Petering & Shih (1975) have calculated from Leichter's data a second-order rate constant of $6{\cdot}4 \times 10^{-3}$/M. sec for the sulphitolysis of thiamine at pH 7 and 25°C.

Sulphitolysis of disulphide bonds. Sulphite can reversibly lyse disulphide bonds by a nucleophilic displacement mechanism resulting in the formation of thiol and *S*-sulphonate compounds (Cecil, 1963). Under non-denaturing conditions and at physiological pH, the reaction between sulphite and S–S bonds in free cystine goes essentially to completion (cysteine *S*-sulphonate is stable at this pH) while the disulphides of most proteins are unreactive. The unreactive protein disulphide bonds are apparently protected from nucleophilic attack either sterically or by the unfavourable electronic environment of neighbouring amino acids. Quantitative reaction of all disulphide bonds in a specific protein can be accomplished by denaturing the protein and using high concentrations of sulphite reactant.

McArdle (1967) demonstrated the reaction of sulphite with non-mercaptalbumin at physiological pH, producing an *S*-sulphonate compound at the albumin–cysteine (or albumin–glutathione) site. Gregory (1981) has recently confirmed this observation using both purified rabbit-plasma albumin and fresh whole rabbit plasma. Gregory further demonstrated, at physiological pH, the partial sulphitolysis of disulphides present in rabbit-plasma fibronectin protein. Gunnison & Palmes (1978) and Gunnison & Benton (1971) have shown that chemically stable plasma-protein *S*-sulphonate compounds are formed in a matter of minutes or hours during the incubation of sulphite (approximately 0·5 mM) with the plasmas of several species of mammals at physiological pH.

Free-radical reactions

Sulphite can be oxidized to sulphate by free oxygen (autoxidation) *via* a free-radical chain mechanism which is initiated by superoxide-anion ($O_2^{-}\cdot$) or $HSO_3\cdot$ radicals (McCord & Fridovich, 1969; Yang, 1970). Once initiated, chain-propagating reactions generate highly reactive free-radical intermediates such as sulphur–oxygen species (Hayon, Treinin & Wilf, 1972), O_2^{-}, and the peroxide ($HO_2\cdot$) and hydroxyl ($OH\cdot$) radicals. The chain-initiating radical $O_2^{-}\cdot$ can be generated by certain enzymatic reactions and both $O_2^{-}\cdot$ and $HSO_3\cdot$ can be produced by the reaction of transition-metal ions with oxygen (Yang, 1970). Fridovich & Handler (1961) have shown that the catalytic action of several oxidative enzymes (i.e. xanthine oxidase, liver aldehyde oxidase, cytochrome oxidase, lipoxidase and peroxidase) can initiate the aerobic oxidation of sulphite, while diverse other oxidase enzymes cannot. It appears that enzymes capable of initiation are those that effect the univalent reduction of oxygen to produce the superoxide anion.

Free radicals generated by the aerobic oxidation of sulphite initiate several reactions of potential biological significance (Hayatsu, 1976). The rate of these reactions is, in general, enhanced by conditions that favour autoxidation of sulphite, such as the presence of transition-metal ions (e.g. Mn^{++}, Fe^{+++}) and oxygen, and is inhibited by free-radical scavengers (e.g. hydroquinone). Sulphite autoxidation occurs readily at pH 7 and reaction of the free radicals generated with various substrates proceeds more rapidly at sulphite concentrations of approximately 1–20 mM than at higher concentrations. At 1 M, for example, sulphite ions can compete effectively with potential substrates for the free radicals generated by sulphite autoxidation, while at 20 mM this competition by sulphite is relatively ineffective due to low concentration. Conditions favourable for the aerobic oxidation of sulphite (stated above) have been used in investigations of the reactivity of the free-radical intermediates of sulphite autoxidation, and will be referred to in the following paragraphs as a sulphite/free-radical environment.

Significant cleavage of the glycosidic linkages of uridine and cytidine, but not of purine nucleosides or pyrimidine deoxyribonucleosides, occurred in a sulphite/free-radical environment (Kitamura & Hayatsu, 1974). In this same system there was also extensive

fission of the chains of polyuridylic acid [poly(U)] and polycytidylic acid [poly(C)], but not of polyadenylic acid [poly(A)] or poly(U):poly(A). Breaking of DNA phosphodiester linkages was also observed when double-stranded DNA of phage T7 was incubated in a sulphite/free-radical environment and subsequently treated with alkali (Hayatsu & Miller, 1972).

4-Thiouracil and 6-isopentenyladenosine (ipa) are among the minor base constituents of yeast transfer RNAs. Both of these bases are modified as a result of autoxidation of sulphite, probably directly by the sulphite-ion radical ($SO_3^- \cdot$), forming uracil 4-sulphonate and a sulphonated product of ipa, respectively. The site of sulphonate formation in the latter molecule is the unsaturated bond of the isopentenyl side chain and is an example of the addition of the sulphite-ion radical to an olefinic double bond. This class of reaction is described in some detail by Stacey & Harris (1963). The optimum pH for the reaction is in the range 5–7, and the speed of the reaction is highly dependent upon the solubility of the olefin in aqueous medium. Allyl alcohol, for example, readily undergoes addition of sulphite *via* a free-radical mechanism.

Methionine and certain other dialkyl sulphides are oxidized to their respective sulphoxides (R—S—R) in

a sulphite/free-radical environment (Yang, 1970). A reaction scheme has been proposed in which superoxide anion and hydroxyl radicals are responsible for sulphide oxidation. Yang (1973) has also shown that tryptophan is destroyed by free radicals generated during the aerobic oxidation of sulphite.

Kaplan, McJilton & Luchtel (1975) have demonstrated that 0·5 to 10 mM-sulphite can induce the oxidation of a heterogeneous mixture of unsaturated fatty acids contained in corn oil in a dose-dependent fashion, presumably by a free-radical mechanism. Although the oxidation products probably consisted mainly of peroxides, the possible presence of other oxidation products could not be excluded by the analytical method used (i.e. reactivity with thiobarbituric acid, TBA). The precise chemistry of the reactions taking place was not investigated. The mechanism of the oxidative reaction(s) was not clear since the formation of TBA-reactive materials was inhibited by the presence of an antioxidant, indicating a free-radical mechanism, and yet Mn^{++} also inhibited rather than enhanced the reaction, suggesting that sulphite autoxidation was unimportant.

Similar results were obtained by Inouye, Ikeda, Ishida, Ogata, Akiyama & Utsumi (1978), who measured lipid peroxidation (TBA-reactive material) in rat-liver homogenate. Addition of 2 mM-sulphite to the homogenate greatly increased the TBA-reactive material while addition of 2 mM-Mn^{++}, even in the presence of sulphite, suppressed it.

In vitro modification of enzyme activity

Sulphite inhibits the *in vitro* activity of several enzymes with either NAD or flavin nucleotide cofactors by adding to the active site of the cofactor, as previously discussed. G. Pfleiderer, D. Jeckel & T. Wieland, in 1956, first postulated an enzymatically inactive complex between lactate dehydrogenase (LDH), NAD^+ and sulphite (from Ciaccio, 1966). Parker, Lodola & Holbrook (1978) estimated the dissociation constant for this complex ($LDH\text{–}NAD^+\text{–}SO_3^{--} \rightleftharpoons SO_3^{--} + LDH\text{–}NAD^+$) to be approximately 10^{-7} M (as compared to $1{\cdot}5 \times 10^{-2}$ M for the non-enzymatic adduct) and concluded that the binding of NAD^+ to LDH activated the nicotinamide ring for attack by sulphite by a factor of approximately 10^5. The effect on the activities of LDH and of several other NAD^+-dependent dehydrogenases (malate, alcohol, glutamate and α-glycerophosphate) is apparent from the *in vitro* experiments of Ciaccio (1966) in which 50% inhibition was observed in the presence of 0·03–0·5 mM-sulphite.

The sulphite adducts of flavins bound to enzymes also inactivate the enzymes and exhibit greatly enhanced stabilities relative to adducts of free FAD, FMN and model isoalloxazines. A prime example is the binding of sulphite to FAD–glucose oxidase. The dissociation constant of this complex is approximately 7×10^{-4} M at pH 7 (Swoboda & Massey, 1966) compared to dissociation constants of approximately 2 M for sulphite adducts of free flavin. Although the FAD–sulphite adduct of glucose oxidase exhibits considerable stability in the presence of sulphite, it is, nevertheless, unstable in the absence of sulphite, as was demonstrated by the regeneration of active enzyme by dialysis. This reversibility is an important property with regard to the toxicological significance of the sulphite adducts of flavins (and NAD). Massey, Müller, Feldberg, Schuman, Sullivan, Howell, Mayhew, Matthews & Foust (1969) have catalogued a number of flavoproteins in terms of their reactivity with sulphite, i.e. the formation of flavin nucleotide–sulphite adducts. A series of oxidase enzymes (D- and L-amino acid oxidase, oxynitrilase, lactate oxidase and glycollate oxidase) reacted readily with sulphite, forming complexes with dissociation constants ranging from 10^{-3} to 10^{-7} M. Presumably the complexed enzymes were inactive although this was not measured. It should be noted that the flavoprotein dehydrogenases tested did not form adducts even when incubated in 20 mM-sulphite for several hours.

Incubation of cytochrome oxidase with 0·5 or 5 mM-sulphite at pH 7 for 4 hr inhibited its activity by 37 and 80% respectively (Cooperstein, 1963). The mechanism of inhibition was believed to involve one or more disulphide bonds, since other disulphide bond-reducing agents, such as cysteine and reduced glutathione, were also inhibitory. The inhibition could be reversed by incubation with oxidized glutathione.

α-Glucan phosphorylase (α-1,4-glucan:orthophosphate glucosyltransferase; EC 2.4.1.1) from rabbit muscle, which catalyses the reversible formation of glucose 1-phosphate from glycogen, is substantially inhibited at pH 6 by sulphite concentrations in the 10–30 mM range (Kamogawa & Fukui, 1973). This inhibitory effect is highly specific and completely reversible by dialysis. Sulphite acts as a competitive inhibitor (K_i = 7 mM) with respect to glucose 1-phosphate (K_m = 11 mM) in glycogen synthesis and with respect to inorganic phosphate (P_i) in glycogen degradation. The authors speculate that this competition for the phosphate-binding site of the enzyme may be due to the structural similarity of HSO_3^- and phosphate.

Harkness & Roth (1969) have reported a striking

sulphite-induced enhancement of activity of 2,3-diphosphoglyceric acid (2,3-DPG) phosphatase. This enzyme is believed by these authors to be the physiological phosphatase catalysing the conversion of 2,3-DPG to 3-phosphoglyceric acid and P_i. Since 2,3-DPG plays a major role in regulation of oxygen affinity for haemoglobin, any factor that affects its concentration in the red cell could be of physiological importance. In the experiments of Harkness & Roth (1969) incubation of the purified phosphatase enzyme with 20 mM sulphite for 1 hr at pH 7·8 and 37°C produced a 37-fold increase in enzyme activity; at 2·5 mM-sulphite, activity was increased by about 15-fold. Incubations with lower concentrations were not performed.

Sulphite is a potent inhibitor of most sulphatase enzymes (Roy, 1960). For example, the K_i values for sulphite inhibition of aryl sulphatases A, B and C from ox liver are 2 μM, 0·5 mM and 0·1 mM, respectively. If precautions are taken to minimize the autoxidation of sulphite, then K_i for inhibition of sulphatase A decreases to approximately 0·2 μM (Roy, 1976). The early work on the kinetics of aryl sulphatases was performed with nitrocatechol sulphate and other unphysiological substrates without certain knowledge of the true physiological substrates. More recently, the physiological substrates for the sulphatases have been thought to include lipids containing galactosyl 3-sulphate residues (such as cerebroside sulphate), mucopolysaccharides containing *N*-acetylgalactosamine 4-sulphate residues (such as dermatan sulphate), heparin sulphate, chondroitin sulphates and steroid sulphates (Roy, 1976).

Sulphite toxicity in biologically active, *in vitro* test systems

Modification of activities of RNA, DNA and associated proteins

Shapiro & Braverman (1972) have demonstrated that conversion of uracil to the 6-sulphonate adduct interferes with hydrogen binding to adenine and reduces the ability of poly(U) to form a helical complex with poly(A). The uracil–sulphonate adducts were formed in poly(U) by reaction with 1 M-sulphite at pH 7, followed by stabilization at pH 4 and dialysis to remove excess sulphite. The modification of uracil residues of poly(U) also inhibited its ability to code for phenylalanine incorporation into protein in an *Escherichia coli* cell-free protein-synthesizing system operating at approximately physiological pH. A relatively small percentage of adduct formation (2·6%) caused a much larger decrease in incorporation (54%), leading the authors to suggest that a single uracil saturation might be sufficient to block translation at that point. This same research group later showed that sulphite could similarly modify natural messenger RNA (from coliphage MS2) and ribosomal RNA from *E. coli*, leading to decreases in the incorporation of amino acids into protein (Braverman, Shapiro & Szer, 1975).

In related experiments, uracil–sulphonate adducts formed in calf thymus DNA by deamination of cytosine residues, interfered with the DNA polymerase reaction, thus inactivating DNA as a template (Kai, Tsuruo & Hayatsu, 1974). Treatment with alkali, which removed sulphite from the dihydro-6-sulphonate moiety, restored the template activity of DNA. The modified DNA was prepared by heat denaturation and reaction with approximately 1 M-sulphite at pH 6. In experiments in which calf-thymus DNA was not heat-denatured (Shapiro, Braverman, Louis & Servis, 1973), sulphite did not convert residues of cytosine to uracil, implying that single-stranded DNA is a requisite for this reaction.

Sulphite has been shown to inhibit the transforming activity of DNA isolated from a bacterium (strains of *Bacillus subtilis*) under conditions that favour the aerobic oxidation of sulphite, suggesting a free radical-mediated mechanism of DNA alteration (Inoue, Hayatsu & Tanooka, 1972). Inhibition was greatest at approximately 20 mM-sulphite and decreased progressively as the sulphite concentration increased to 1 M. Other factors that decreased the rate of sulphite autoxidation, such as free-radical scavengers and elimination of oxygen, also decreased the sulphite-mediated inhibition of transforming activity. Inactivation of the DNA transforming activity was strongly inhibited by 4-thiouridine which is known to react with the sulphite-ion radical. Thus, this radical was assumed to be the species primarily responsible for the inactivation.

A free-radical mechanism was also implicated in the sulphite-mediated inactivation of bacteriophage lambda (Kudo, Miura & Hayatsu, 1978). Inactivation of phage infectivity of indicator bacteria was observed in sulphite concentrations of 0·1–10 mM in incubations at pH 7 and 37°C. After a 4-hr incubation in 10 mM-sulphite, the infectivity of the phage had decreased to 10^{-5} of its initial value. Phage inactivation was attributed not to DNA damage but to alteration of coat proteins; this affected their adhesion of bacteria and their ability to inject DNA. Several lines of evidence suggested that tryptophan in the coat proteins was modified by reaction with the sulphite-ion radical.

Turchinsky, Kusova & Budowski (1974) have demonstrated the sulphite-catalysed crosslinking of the maturation and coat proteins with the nucleic acids of the RNA bacteriophage, MS2. The MS2 phages were treated with 1 M-sulphite at pH 7 for 0·5–4 hr, with consequent covalent association of approximately 1% of the protein with RNA. The mechanism of crosslinking was presumably by transamination of 5,6-dihydrocytosine-6-sulphonate as discussed in a previous section.

Sklyadneva *et al.* (1979a) have also presented evidence for the sulphite-catalysed transamination of cytosine bases with protein in bacteriophage DNA. These authors propose that the intermediate, 5,6-dihydrocytosine-6-sulphonate, is stabilized *in situ* by polar groups of protein, especially the amino groups of basic amino acids. This stabilization is effective at refrigerator temperatures for up to 4 months. When phage particles are disintegrated, however, the sulphonate adduct in the DNA becomes unstable and, depending upon the specific conditions, either reverts to a cytosine residue or undergoes transamination. This *in situ* stability of cytosine–sulphonate adducts may have important implications for the *in vivo* rate of deamination of cytosine to uracil.

Chromosome damage and mutagenesis

As anticipated from chemical data, sulphite was shown to cause mutations, presumably by deamination of cytosine to uracil. Cultures of several mutant strains of *E. coli* were treated with 1 M-sulphite at pH 5·2 for 30 min and the frequency of back mutation was determined (Mukai, Hawryluk & Shapiro, 1970). Only those mutants that were cytosine–guanidine (C:G) at the mutant site showed an increase in reversion frequency. When incubations were performed at pH 7 or 8, sulphite had no measurable effect on reversion frequency, a result consistent with chemical data on the pH profile for deamination.

A similar specificity for C:G to A:T (adenine–thymine) transitions was reported by Summers & Drake (1971) using bacteriophage T4rII as a test system (although T4 contains the cytosine analogue 5-hydroxymethylcytosine). At pH 5, inactivation and mutation frequency of the phage showed excellent dose-response relationships with both sulphite concentration (0·2–0·9 M) and treatment time. The reversion frequency resulting from a 4-hr treatment with 0·9 M-sulphite was approximately $110/10^7$. Recent duplication of these experiments, however, revealed the initial findings to be in error. Sulphite was not capable of causing a measurable rate of reversion of T4 phage, although a 10–20-fold lower mutation rate than that initially reported could not be excluded (J. W. Drake, personal communication 1981).

An increase in mutation frequency of phage lambda incubated in 3 M-sulphite at pH 5·6 was observed by Hayatsu & Miura (1970). Maximum mutation frequency was produced after 1·5 hr, while inactivation of the phage continued for the 3 hr duration of the experiment.

In his review of the genetic effects of sulphite, Shapiro (1977) cites two reports in which sulphite apparently caused mutations in *Saccharomyces cerevisiae* and *Micrococcus aureus* at much lower concentrations than are usually required (i.e. 5 and 10 mM, respectively). In the former case, however, a very low pH (3·6) was required.

In the above experiments, sulphite-induced mutagenicity was observed in cells containing double-stranded DNA. This appears to be inconsistent with other experiments in which the cytosine bases in isolated double-stranded DNA were inert to sulphite (Shapiro, 1977; Shapiro *et al.* 1973). In reality, however, DNA, especially in certain growth phases, always exists partially in its reactive single-stranded form (Bjursell, Gussander & Lindahl, 1979).

The proposed mechanism of sulphite-induced mutagenesis, that is conversion of cytosine to uracil, is not consistent with the existence of uracil-DNA glycosidase. This enzyme, discovered initially in *E. coli* (Lindahl, 1974), catalyses the excision from DNA of uracil bases produced by deamination of cytosine, thus apparently preventing C:G to A:T transition. Recent data suggest that sulphite-induced mutations at C:G sites actually involve deamination of 5-methylcytosine to thymine. Since the newly-formed thymine in DNA is not recognized by a glycosidase, it cannot be excised and repaired. Coulondre, Miller, Farabaugh & Gilbert (1978) have demonstrated that 5-methylcytosine residues in *E. coli* are associated with a high rate of spontaneous mutation consisting of C:G to A:T transitions (i.e. hot spots). Furthermore, in a strain of *E. coli* that lacks uracil-DNA glycosidase, the rate of spontaneous transitions at cytosine residues is elevated to the rate observed at 5-methylcytosine residues (Duncan & Miller, 1980).

On the other hand, Wang, Gehrke & Ehrlich (1980) have recently demonstrated that, at pH 5·5 and 3 M-sulphite, under conditions where more than 96% of cytosine residues in single-stranded DNA were converted to uracil, only 2–3% conversion of 5-methylcytosine residues to thymine occurred. Wang & Ehrlich (1980) concluded that it is much more likely that cytosine rather than 5-methylcytosine residues are involved in sulphite-induced mutagenesis. This point is the subject of continuing research and is still open to question. Preliminary experimentation necessary for calculating the rate of 5-methylcytosine deamination under physiological conditions is now underway in the laboratory of Dr R. Shapiro (personal communication, 1980).

Recently, Mallon & Rossman (1981) have demonstrated an enhancement of UV mutagenicity resulting from exposure at physiological pH to much lower sulphite concentrations than are required for measurable conversion of cytosine to uracil. Cells from a Chinese hamster line, V79, exposed to 10 mM-sulphite at pH 7·4, either during or immediately following UV irradiation, showed an approximately twofold increase in mutation frequency over that caused by UV treatment alone. In similar experiments with *E. coli*, 100 mM-sulphite caused an eight-fold increase. In both cases, exposure to sulphite alone had no effect on mutation frequency. Since the co-mutagenic effect of sulphite was essentially of equal potency whether sulphite exposure occurred during or immediately following UV irradiation, Mallon & Rossman (1981) speculated that sulphite might have been affecting a DNA repair process. Subsequent experiments utilizing two *E. coli* strains deficient in the excision repair process demonstrated that the presence of sulphite did not enhance UV mutagenesis, implying that the function of sulphite in the initial experiments was to inhibit excision repair.

Perry & Evans (1975) have demonstrated a positive correlation between mutagenesis and sister chromatid exchange and suggest the latter as a highly sensitive technique for assaying the chromosome mutagenicity of environmental agents. MacRae & Stich (1979) showed that sulphite induces dose-related sister chromatid exchange in Chinese hamster ovary cells at concentrations between approximately 0·03 and 7 mM. The potency of this induction, however, was much lower than that exhibited by the strong mutagenic agents shown to possess this ability. MacRae & Stich (1979) suggested that cleavage of the DNA chain by free radicals, probably hydroxyl radicals, generated by autoxidation of sulphite (Hayatsu & Miller, 1972) might be responsible for this action of sulphite.

In a different type of assay, damage to the chromosomes of mammalian oocytes was observed following *in vitro* exposure to sulphite (Jagiello, Lin & Ducayen, 1975). In the same experiments there was also an inhibition of entry of oocytes into meiosis when they were cultured in the presence of sulphite. Mouse oocytes, in general, were more sensitive than those of the cow or ewe and when exposed to sulphite concentrations of

approximately 0·2 mM or above showed a slight inhibition of entry into meiosis which became complete at a sulphite concentration of 6 mM. Chromosome 'fuzziness' also occurred at 0·2 mM and higher concentrations, but the authors ascribed no genetic significance to this type of aberration. More significant genetically was the fragmentation of chromosomes and the anaphase lagging that occurred sporadically in cow and ewe oocytes at concentrations of sulphite of approximately 3 mM and above.

Effects on mammalian cells in culture

The adhesion of cultured Chinese hamster cells (cell line Don) to the substratum was slightly inhibited by incubation with 10 mM-sulphite and severely inhibited by 30 or 50 mM-sulphite (Kudo, Hayatsu, Yokoiyama & Kuroda, 1980). A possible explanation for this observation has been offered by Gregory (1981) who found that sulphite concentrations at or above 30 mM progressively cleaved disulphide-linked rabbit-plasma fibronectin dimers into monomers. A plasma fibronectin-type protein is also present in disulphide-bonded aggregates in relatively large quantities on the surface of cells (cell surface protein; CSP) where it functions in cell–substratum adhesion and cell–cell interactions. Disruption of CSP by strong disulphide reducing agents has been shown to destroy its functions (Ali & Hynes, 1978).

An observation by Kikugawa & Iizuka (1972) that 7·5 mM-sulphite inhibits ADP- and collagen-induced aggregation of rabbit platelets may well be linked to the above finding of Kudo *et al.* (1980), by similar underlying mechanisms.

Thompson & Pace (1962) measured cell proliferation in mouse fibroblasts, mouse-liver cells and HeLa cells exposed in culture to initial sulphite concentrations ranging from approximately 1 to 20 mM for periods up to 9 days. There was complete inhibition of growth in all cell lines cultured in the high sulphite concentration, while HeLa cells, which were the most sensitive to sulphite, showed marked growth inhibition even at 1 mM-sulphite. Results consistent with these findings were obtained by Das & Runeckles (1974), although synchronous cultures of *Chlorella pyrenoidosa* were used. In these studies, exposure of cells to initial sulphite concentrations of 0·5–2 mM for a period of 48 hr caused a progressive decrease in cell number and in DNA content, expressed as a percentage of dry weight, but not in RNA or protein content. During the 48-hr exposure period, there was a decline in pH from 6·6 to approximately 4 (measured in a culture containing about 2 mM-sulphite) which may have affected entry of sulphite into the cell. The authors concluded that sulphite affected active growth of the cells by impairing DNA synthesis.

Inhibition of DNA synthesis (measured by [^{3}H]thymidine incorporation) was also observed by Chin, Bissell & Bassham (1977) in chick-embryo fibroblasts cultured in the presence of sulphite for 18 hr. While 0·05 mM-sulphite caused no measurable inhibition, 0·1 and 1·0 mM-sulphite caused a 14 and 52% reduction in thymidine incorporation, respectively. After incubation for 48 hr, decreased cell viability was observed in 0·5 and 1·0 mM-sulphite. Sulphite did not affect cell-membrane permeability to mannitol (passive diffusion) or to 2-deoxyglucose (carrier-mediated transport). In addition, no discernible effect of sulphite on glucose metabolism was revealed by monitoring selected intermediates of the glycolysis pathway, glycogen synthesis, the pentose shunt and the tricarboxylic acid cycle.

Timson (1973) cultured human lymphocytes in 0·1–10 mM-sulphite and concluded that exposure to 10 mM-sulphite for 72 hr was cytotoxic, while lower concentrations had an antimitotic effect which was possibly due to inhibition of DNA synthesis during the early stages of mitosis. In this system, exposure to 0·1 mM-sulphite over a period of 72 hr caused 43% inhibition of mitosis, and a similar exposure to 1·0 mM-sulphite produced a 63% inhibition.

In a perplexing study which is somewhat difficult to relate to others of its kind, Schneider & Calkins (1970) exposed human lymphocytes in culture to sulphite by bubbling SO_2 through the medium. Although the sulphite concentration was not measured either at the onset of exposure or during the incubation period, which lasted for up to 3 days, it is possible to calculate from the information given that the initial concentration was approximately 0·005 mM, assuming complete absorption of SO_2 by the medium. The pH during the incubation period varied between approximately 6·6 and 7·2. There was a significant decrease both in DNA synthesis, as measured by the percentage of cells that incorporated [^{3}H]thymidine, and in the mitotic index of cells exposed to sulphite in comparison with appropriate control cells. These effects were most apparent in cells exposed before DNA synthesis was initiated. In addition, chromosomal abnormalities, consisting mainly of a reduction in number and in clumping and fuzziness, occurred at a higher incidence in the sulphite-exposed cultures.

These experiments demonstrated detrimental effects at an estimated concentration of sulphite approximately two orders of magnitude below those reported by other investigators as causing similar changes. It is felt, however, that the results of Schneider & Calkins (1970) are open to question because the authors neglected to test other concentrations of sulphite to demonstrate conclusively that the effects observed were truly a function of sulphite exposure. The delivery of the sulphite dose by bubbling SO_2 through the medium introduced the possibility of side effects due to physical damage of cells and/or oxygenation of the culture medium. Although these side effects were supposedly controlled for in cultures that received air only, the possibility of a synergistic effect between sulphite and the bubbling of air through the medium was not considered.

A 50% reduction of the intracellular concentration of 2,3-DPG in human erythrocytes resulted from incubation of the cells for 4 hr with 4 mM-sulphite at pH 7·5 and 37°C (Parker, 1969). Essentially no intracellular 2,3-DPG remained when the concentration of sulphite was increased to 20 mM. These data can be explained by the sulphite-induced activation of 2,3-DPG phosphatase of human erythrocytes discussed previously (Harkness & Roth, 1969). The depletion of 2,3-DPG from erythrocytes was accompanied by a reversible increase in their passive permeability to Na and K ions, a change that was thought to be a direct effect of sulphite and not to be caused indirectly by 2,3-DPG levels.

Mammalian toxicity

Chronic sulphite feeding studies

In studies in which sulphite was administered to animals (usually rats) in the diet or drinking-water, the concentration of sulphite was often expressed in different units, making direct comparisons difficult. Therefore, in this section, an attempt is made to express the sulphite exposure in all experiments in terms of mmol consumed/kg body weight/day. Since some investigators have not given the data on food or water consumption required for these calculations, it has been assumed where necessary that rats ingest 100 ml water and 67 g solid diet/kg body weight/day. Further, the instability of sulphite added to the diet or drinking-water has been noted and taken into account by some investigators in their calculations of intake, and ignored by others. No attempt is made here to adjust intake figures for this variable unless adequate data on stability have been given.

It is well documented that sulphite, when pre-mixed with the diet, can cleave the thiamine molecules contained therein and destroy their activity, thereby causing a deficiency of thiamine in the organism (Bhagat & Lockett, 1964; Fitzhugh, Knudsen & Nelson, 1946). Thiamine deficiency has not resulted, however, when sulphite has been administered in fluids, although there is evidence that thiamine can be destroyed in the stomach when ingested simultaneously with sulphite (Lhuissier, 1966). Furthermore, the possibility that sulphite may inhibit the synthesis of thiamine by the bacterial flora of the intestine cannot be excluded (Cremer & Hötzel, 1966). In spite of this destruction by sulphite, recent data show that thiamine is not destroyed systemically by sulphite (Gunnison, Dulak, Chiang, Zaccardi & Farruggella, 1981a).

One of the earliest attempts to investigate the chronic toxicity of sulphite comprehensively was published in 1946 by Fitzhugh *et al.* These investigators administered sulphite to rats for approximately 1 yr by incorporating it into their diet at several concentrations, resulting in nominal intakes in the treatment groups ranging from 0·08 to 13 mmol/kg/day. Unfortunately, these estimates of sulphite intake are meaningful only as ceiling values, since sulphited food was sometimes left in feeder cups for up to a week between changes, during which time as much as 75% of the sulphite was lost due to chemical reaction. In these experiments the investigators attempted, not entirely successfully, to separate the effects of sulphite-induced thiamine deficiency from those due to the ageing of the sulphited diet and to the direct toxicity of sulphite. They concluded that, in addition to the toxic signs attributable to thiamine deficiency, such as polyneuritis, the stunting of growth and atrophy of organs (the latter two due to inanition), other toxic changes were produced by ingestion of the sulphited diets. At a nominal sulphite intake of 1·6 mmol/kg/day or more, the growth rate and average survival time of rats was decreased and pathological changes including gastric squamous epithelial hyperplasia, bleached incisor teeth, brown uteri, calcified renal tubular casts and atrophy of bone and bone marrow were observed. It was not clear, however, which of these signs could be prevented or mitigated by thiamine therapy, or whether residual toxicity was caused by the interaction of sulphite with constituents of the diet and/or the direct action of sulphite on the organism. In 1960, Lockett & Natoff, administering sulphite chronically to rats in their drinking-water at an intake rate of approximately 1–2 mmol/kg/day, observed none of the toxic signs listed by Fitzhugh *et al.* (1946). Their obvious conclusion, therefore, was that sulphite was not directly responsible for this toxicity.

Bhagat & Lockett (1964) later attempted to clarify the roles of thiamine destruction and storage of diet in the development of toxicity resulting from feeding sulphited diets to rats. In these experiments, the growth rate of young rats over a 5–7-wk period was used as an assay for toxicity. Diets contained approximately 130 μg thiamine/100 g and 0·6% sodium metabisulphite at the time of mixing (equivalent to an intake of approximately 5–9 mmol sulphite/kg/day, assuming no loss of sulphite prior to use). However, subsequent storage at room temperature resulted in the rapid and parallel destruction of both sulphite and thiamine content, and after 8 days only approximately 20% of their initial concentrations remained. Young rats fed this diet within 2 months of its preparation showed a decreased growth rate, which could be corrected by thiamine supplementation in spite of weak antithiamine activity resulting from the exposure of dietary yeast to sulphite. Diets that were stored at room temperature for 75 days or longer caused toxicity in the form of a reduced growth rate and diarrhoea, which could not be completely corrected by thiamine supplementation. Although residual sulphite concentrations in the diets at the time of consumption were not measured, it seems almost certain that the sulphite would have been too low after 75 days of storage to be a factor in the development of the observed toxicity.

The most thorough investigation of chronic sulphite toxicity to date is the work of Til, Feron & de Groot (1972a) and Til, Feron, de Groot & van der Wal (1972b) using rats and pigs. Sulphite was administered in the diet, and losses due to chemical reaction prior to feeding were kept to a minimum by frequent diet preparation and storage at low temperature. The loss of sulphite that did occur was measured and the dietary concentrations were corrected accordingly. In addition, thiamine was added to the diet to compensate for its sulphite-mediated destruction, thus ensuring that any toxicity observed during the experiment would not be due to thiamine deficiency.

Sulphite was administered to three generations of rats for periods up to 2 yr at intake rates of approximately 0·7, 1·5, 3, 6, and 13 mmol/kg/day for the five treatment groups, and the health of these animals was compared with that of a matched control group receiving the same diet with no added sulphite (Til *et al.* 1972a). Slight growth retardation was observed in the F_1- and F_2-generation rats of the high-dose group and marginally reduced haematocrit, haemoglobin and erythrocyte counts also occurred in F_0 rats at this treatment level. Occult blood was present in the faeces of approximately 20–50% of rats ingesting 6 mmol/kg/day. Abnormal morphology of the forestomach was observed in some F_2-generation rats ingesting 3 mmol/kg/day, and in rats of the two highest treatment groups more severe hyperplasia and inflammation of both the fore- and glandular stomach

was observed. There were no dose-related trends in tumour frequencies among F_0- and F_1-generation rats that died during the experiment or were killed at 2 yr of age. Likewise, sulphite had no effect on the indices of reproduction and early development investigated, i.e. on fertility (% of females with litters), mean number of pups per litter, birth weight and mortality prior to weaning. In F_2 generation females, kidney weight relative to body weight was increased in the high dosage group, although kidney function, as measured by phenol red excretion, urine specific gravity and glutamic–oxalacetic transaminase activity in the urine, was not adversely affected by sulphite feeding. Increased relative weights of kidneys have also been observed in other chronic and subchronic sulphite toxicity studies. The no-effect level determined from the experiments of Til *et al.* (1972a) was equivalent to an intake of 1·5 mmol/kg/day. This level of consumption, adjusted by a 100-fold safety factor, has been adopted by WHO as its maximum acceptable daily intake (ADI) level, i.e. 0·70 mg (as SO_2)/kg body weight (Joint FAO/WHO Expert Committee on Food Additives, 1974).

In a companion experiment to the one described above, Dutch Landrace pigs were fed sulphite from weaning for periods of up to 48 continuous weeks (Til *et al.* 1972b). The same nominal concentrations of dietary sulphite were used for these animals as for the rats, but actual intakes were considerably less, approximately 0·1, 0·3, 0·6, 1·6 and 3·6 mmol/kg/day in the five treatment groups. The thiamine added to the diet was sufficient to prevent deficiency in all groups except that on the highest dose, in which a slight reduction in hepatic thiamine level occurred. Growth and food consumption were significantly decreased only in the highest dose group (3·6 mmol/kg/day). Paired-feeding studies showed that this decreased rate of growth was due solely to decreased food consumption and was not a direct cause of ingested sulphite. Increases in the relative weights of liver and kidney in this same treatment group, however, were attributable to sulphite consumption, but were not accompanied by histological changes. As in the experiment with rats, the most damaging effects of sulphite were the inflammatory and hyperplastic changes in the stomach mucosa of animals in the two highest dosage groups, although no occult blood was found in the faeces of these animals.

Several other chronic feeding studies of merit conducted in rats will not be reviewed here in any depth (Cluzan, Causeret & Hugot, 1965; Lanteaume, Ramel, Girard, Jaulmes, Gasq & Ranau, 1965; Lockett & Natoff, 1960). In all of these studies sulphite was either added to the drinking-water or given in solution by gastric intubation, resulting in daily intakes of between 0·05 and 2 mmol/kg. Usually several generations of rats were treated and all experiments lasted for at least 1 yr. In these studies, investigators measured the effect of sulphite on such parameters as fertility, the general health and growth rate of offspring, organ weights, haematology, food intake and growth rate of adults, histological appearance of major organs and tumour incidence. In general, no consistent trends attributable to sulphite exposure were apparent in any of these parameters.

Short-term studies

Investigations by Til *et al.* (1972a) of the toxicity of high doses of sulphite ingested with the diet over relatively short periods, showed that the food intake, food efficiency and growth rate were drastically reduced in young male rats ingesting approximately 50 mmol sulphite/kg/day for 8 wk. In addition, severe anaemia, increased spleen weight and a slightly increased leucocyte count were observed after only 3 wk. Gunnison *et al.* (1981a) have confirmed these observations and have shown that the anaemia results not from the systemic activity of sulphite following ingestion but from the interaction of sulphite with a constituent(s) of the diet, possibly cyanocobalamine.

In other short-term studies of up to 4 months duration, daily sulphite intakes ranging from approximately 0·5 to 6 mmol/kg caused a slight increase in the excretion of calcium and had no effect on the hepatic stores of vitamin A (Joint FAO/WHO Expert Committee on Food Additives, 1974; Lanteaume, Morin, Palluel & Pallaget, 1978). The effect of sulphite on calcium excretion is probably of little or no physiological significance.

In thiamine-deficient rats, small amounts of sulphite administered separately from the diet depressed weight gain and survival, while in rats not deficient in thiamine, sulphite intake up to 6·2 mmol/kg/day had no effect on weight gain over a 3-month period (Cremer & Hötzel, 1966). This observation may help to explain the toxicity of sulphite reported by Fitzhugh *et al.* (1946) and discussed earlier. In contrast to the rat data, approximately 0·1 mmol sulphite/kg/day administered in imbibed fluids for 25 consecutive days to human volunteers with thiamine deficiency (determined by biochemical signs) caused no clinical, neurophysiological or biochemical alterations compared with controls (Cremer & Hötzel, 1970). The bulk of evidence accumulated from both long- and short-term studies supports the view that sulphite administered in fluids separately from the solid diet does not measurably reduce the thiamine status of the animal.

In spite of evidence of sulphite-induced chromosome aberrations resulting from *in vitro* exposure (see previous section), sulphite did not induce a detectable increase in dominant-lethal mutations in either male or female germ cells of mice receiving repeated daily intraperitoneal injections of 2·9–4·8 mmol/kg (Generoso, Huff & Cain, 1978). Nor were Jagiello *et al.* (1975) successful in inducing chromosome aberrations in mouse oocytes cultured *in vitro* following a single intravenous injection of up to approximately 2 mmol sulphite/kg.

Reviewing the data from experiments designed to evaluate the mammalian toxicity of ingested sulphites leads to the conclusion that apart from the indirect toxicity resulting from destruction of dietary thiamine or other changes in the diet, and the direct irritant effect on the gastro-intestinal tract at relatively high intake levels, no serious adverse effects were observed as a result of chronically administered sulphite. This conclusion is surprising in the light of the reactivity of sulphite with many biologically important molecules and the toxicity of sulphite observed in biologically active *in vitro* test systems. The difference between the toxic potential of sulphite perceived from *in vitro* data

and the toxicity observed in *in vivo* experiments has been noted previously (Shapiro, 1977); the probable explanation becomes apparent with an understanding of sulphite metabolism.

Mammalian sulphite metabolism

The primary route of sulphite metabolism in mammals is its enzymatically mediated oxidation to sulphate. The enzyme involved, sulphite:cytochrome *c* oxidoreductase (EC 1.8.3.1), termed sulphite oxidase, is apparently ubiquitous among mammalian species and is present at high levels in the liver and in lower concentrations in most of the other tissues of the body. Sulphite oxidase, located in the mitochondrial intermembranous space, exists as a dimer of identical subunits, each consisting of a molybdenum ion (Mo^{6+}) and a haem molecule in addition to the apoenzyme. The *in vivo* oxidation of sulphite involves the transfer of a pair of electrons from sulphite to the Mo^{6+} ions and then to the haems associated with the enzyme molecule. The electron pair is then passed to cytochrome *c* of the respiratory chain, eventually reducing 0·5 O_2 to H_2O and producing 1 molecule of ATP in the process. Although sulphite can be autoxidized by a free-radical chain mechanism under appropriate conditions, it is thought that this reaction sequence does not proceed readily in mammalian tissues for a variety of reasons (Cohen & Fridovich, 1971).

In addition to being the major metabolic pathway of exogenous sulphite (ingested sulphite and inhaled SO_2), enzymatically mediated oxidation of sulphite to sulphate is the terminal step in the catabolism of sulphur-containing amino acids. Thus, an enzyme that apparently evolved to protect the tissues of the body from the insult of endogenously-produced sulphite also functions in the metabolism (and presumably the detoxification) of this same substance originating from exogenous sources. Assuming that the sulphate excreted by animals in 'sulphur balance' originates primarily from the catabolism of sulphur-containing amino acids, it can be estimated from data on sulphate excretion that the daily quantity of endogenous sulphite generated by humans is approximately 0·3–0·4 mmol/kg (Institute of Food Technologists and Committee on Public Information, 1976). Under normal circumstances this is considerably greater than the estimated intake of exogenous sulphite (see Introduction).

The capacity of mammalian sulphite oxidase for sulphite oxidation is extremely high compared with the normal sulphite load from endogenous and exogenous sources. For example, Cohen, Drew, Johnson & Rajagopalan (1973) have estimated by *in vitro* assay that the sulphite oxidase contained in the tissues of the rat is theoretically capable of oxidizing sulphite at the rate of approximately 750 mmol/kg/day. Also, Oshino & Chance (1975) and Wilkins, Greene & Weller (1968) demonstrated that the perfused livers of rats and dogs can oxidize sulphite for short periods of time at rates of at least 58 and 18 mmol/kg/day, respectively. Using established pharmacokinetic techniques, Gunnison, Bresnahan & Palmes (1977) investigated the rate of sulphite oxidation in intact rats following rapid intravenous delivery. Elimination of sulphite, which occurred predominantly by metabolism to sulphate, was characterized by first-order rate constants of the order of 0·7–1/min which is equivalent to a half-life for sulphite of approximately 1 min. Further, Gibson & Strong (1973) were unable to detect sulphite in the urine of rats following administration of approximately 6 mmol/kg by gastric intubation. Since sulphite is readily absorbed from the gastro-intestinal tract (Bhagat & Lockett, 1960), this observation attests to the capacity of rats to metabolize systemic sulphite rapidly.

Gunnison *et al.* (1977) compared the activity of sulphite oxidase in rats with that in rabbits and rhesus monkeys using *in vivo* kinetic methods, and demonstrated that rats exhibit approximately three and five times greater activity, respectively, than the latter two species. In addition, Johnson & Rajagopalan (1976a,b) have shown by *in vitro* assay that rat liver possesses approximately 10–20 times more sulphite oxidase activity than does human liver. These comparisons suggest that the rat may be a poor species for the evaluation of sulphite toxicity in humans since the opportunity for potentially damaging reactions of sulphite is comparatively less in the rat due to more rapid metabolism of sulphite to sulphate.

Although the rapid rate of oxidation of sulphite by sulphite oxidase certainly minimizes the quantitative importance of other metabolic pathways, rats injected intraperitoneally with approximately 3 mmol/kg/day and rabbits and rhesus monkeys ingesting approximately 2 mmol/kg/day nevertheless metabolized a portion of this exogenous sulphite to *S*-sulphonate compounds in the plasma (Gunnison & Palmes, 1978). Indeed, the rats and rabbits possessed detectable concentrations of plasma *S*-sulphonate compounds prior to exposure to exogenous sulphite, indicating that the sulphite generated endogenously from sulphur-containing amino acid catabolism was also partially metabolized *via* this route. Because of their relative biological stability, these *S*-sulphonate metabolites were evident in plasma, which does not usually contain detectable free sulphite.

When sulphite is present in the tissues in sufficiently high concentration, it reacts with β-mercaptopyruvate (a normal intermediate in sulphur-amino acid catabolism) forming inorganic thiosulphate ($S_2O_3^{--}$). This metabolite is, at most, marginally detectable in the urine of normal humans and rats, while in both these species large quantities are excreted into the urine of individuals that are deficient in sulphite oxidase and have, therefore, relatively high systemic levels of sulphite.

Throughout the literature on the investigation of mammalian sulphite toxicity there is an almost total absence of any attempt to determine tissue sulphite concentrations. This seems inexplicable, particularly since concentration is the most logical and appropriate basis for the correlation of findings from *in vivo* experiments with data gathered *in vitro*. The limited data existing on *in vivo* sulphite concentration comes from studies of sulphite metabolism in normal and sulphite oxidase-deficient mammals. Gunnison & Palmes (1973 & 1978) determined free sulphite in the plasma of several species, both prior to and during administration of sulphite. Sulphite originating from endogenous sources was not detectable (i.e. was less than 3 μM) and when exogenous sulphite was adminis-

tered in the drinking-water of rats, rabbits and rhesus monkeys at a rate of approximately 2 mmol/kg/day and of mice at approximately 6 mmol/kg/day, plasma sulphite was detected only in the rhesus monkeys. Of the plasma samples collected from six rhesus monkeys at various times during the light cycle (i.e. primary drinking time), sulphite ranging in concentration from 8 to 67 μM was detected in 10 of 23. Gunnison, Farruggella, Chiang, Dulak, Zaccardi & Birkner (1981b) administered sulphite to rats (2–9 mmol/kg) by gastric intubation and measured plasma-sulphite concentration with respect to time following intubation. Peak concentrations ranged from 70 to 800 μM and detectable concentrations persisted for 1–3 hr depending on the dose.

Free sulphite has been reported in the plasma of a child diagnosed as deficient in sulphite oxidase (Shih, Abroms, Johnson, Carney, Mandell, Robb, Cloherty & Rajagopalan, 1977). The plasma concentration increased from 14 μM to about 130 μM and then decreased to 2 μM when the child's diet was first enriched with cysteine and then restricted in sulphur-amino acid content. In sulphite oxidase-deficient rats possessing approximately 1% of the enzyme activity of normal adults, plasma-sulphite concentrations ranged from undetectable to 60 μM with a mean of 18 μM (Gunnison, *et al.* 1981b).

Gunnison & Farruggella (1979) maintained approximately steady-state plasma-sulphite concentrations in the range of 400–650 μM for up to 6 hr by zero order intravenous infusion of rabbits at a rate of approximately 0·9 mmol/kg/hr. The purpose of these infusions was to investigate the kinetics of *S*-sulphonate formation in the aorta and lung. However, during the course of performing the experiments, it was also learned that rabbits could not survive for longer than 2 or 3 hr when plasma-sulphite levels were maintained in the range of 700–1000 μM (unpublished data).

It is worthy of note that in the majority of the *in vitro* experiments discussed earlier, sulphite concentrations were either close to or exceeded the concentrations shown to be lethal to rabbits, and were, in addition, almost always at least one order of magnitude greater than those observed in animals and humans severely deficient in sulphite oxidase. This fact does not diminish the value of the *in vitro* data since the primary purpose of such experiments is usually to identify, or define more accurately under optimal conditions for their development, potentially toxic changes which may also appear *in vivo* under more prolonged but less severe exposure conditions. Nevertheless, the relationship of sulphite concentrations used *in vitro* to those attainable *in vivo* should be considered in predictions of the likelihood of the occurrence of a particular toxic effect *in vivo*.

Alternative mammalian models for evaluation of sulphite toxicity

A case has been made against the use of the rat for the evaluation of sulphite toxicity in humans because of the lower activity of sulphite oxidase in the latter species. In addition, it is known that a genetic deficiency of this enzyme in humans can lower its activity still further. These cases of severe deficiency result in grave health effects which can lead to death (Irreverre, Mudd, Heizer & Laster, 1967). Although occurrences of extreme deficiency are apparently rare, they do illustrate the crucial role of this enzyme in human health and raise questions concerning the pattern of normal variation in sulphite oxidase activity within the human population, as well as the significance of possible minor (i.e. subclinical) deficiencies of sulphite oxidase on the metabolism of sulphite and ultimately on the development of chronic toxic effects. It is clear that the sulphite oxidase-competent rat cannot be used to investigate these questions.

In the course of their thorough investigations into the nature and functioning of sulphite oxidase, Johnson, Rajagopalan & Cohen (1974) found that rats deficient in this enzyme could be produced by manipulation of the tungsten (W) and Mo content of the diet. In an internal environment of relatively high W and low Mo, W either replaces Mo or prevents its incorporation into newly synthesized apoenzyme molecules, causing these molecules to be inactive (Johnson, Cohen & Rajagopalan, 1974). This results in the progressive loss of sulphite-oxidase activity to a lower steady-state level, the magnitude of which is dependent upon the W:Mo intake ratio. By manipulation of this ratio, steady-state sulphite-oxidase activities can be attained over a wide range (Gunnison *et al.* 1981b). This phenomenon has been exploited in our laboratory where groups of sulphite oxidase-deficient female rats, possessing approximately 1–2% of the activity exhibited by normal female adults, have been characterized metabolically and used to investigate sulphite toxicity (Gunnison *et al.* 1981a,b). These deficient rats are considered to be models for humans having approximately 10% of the sulphite-oxidase activity ascribed to normal individuals. The model has helped to answer some questions regarding the primary toxicity of sulphite, specifically concerning the systemic destruction of thiamine and the development of anaemia, as mentioned previously. More importantly, using this model a serious concern has been raised regarding the possible involvement of sulphite in the aetiology of early breast cancer. A low incidence of mammary adenocarcinoma (4/149) was observed in sulphite oxidase-deficient rats of less than 5 months of age which had been treated with a high W/low Mo regime for only 40–65 days. The tissues of these rats were, of course, exposed to elevated concencentrations of endogenously-generated sulphite. No tumours were found in age-matched controls. Although the difference between the sulphite oxidase-deficient and control groups was not statistically significant, the authors believed that because of the early age at which the tumours developed, they were very likely to be treatment related. If this is true, then the role of excess W and a deficiency of Mo (apart from its effect on sulphite-oxidase activity) in the production of early mammary tumours must be considered in addition to that of systemic sulphite. These factors are germane to the evaluation of any toxic effect observed using this model. The direct toxicity of excess W and of Mo deficiency can be at least partially determined by the use of controls, but the possibility of synergism with sulphite must also be considered and is more difficult to evaluate. In spite of these drawbacks, the sulphite oxidase-deficient rat shows

promise as a model for the evaluation of human sulphite toxicity.

It is believed by this author that sulphite oxidase-competent mammals are adequate models for evaluation of the localized effects of inhaled sulphur dioxide on the upper respiratory tract of humans, in terms of accurately reflecting tissue sulphite concentration. There is recent evidence that the upper airways of the respiratory tract directly exposed to inhaled SO_2 (including the nasal passages, trachea and major bronchi) can build up considerable localized concentrations of sulphite without measurably affecting the overall systemic concentration of sulphite (Gunnison, Zaccardi, Dulak & Chiang, 1981). It is probable that the capacity of the animal to oxidize sulphite enzymatically has little bearing on the concentrations of sulphite that develop in the upper respiratory tract of animals inhaling SO_2. Although the voluminous literature on the potential toxicology of inhaled SO_2 is, in general, not within the scope of this review, an important chronic study involving SO_2 will be discussed here because it suggests an active role for sulphite in the origin of bronchogenic tumours. Without citing particular references, it is accurate to state that numerous investigations in a variety of mammals exposed chronically to concentrations of SO_2 several times greater than those ordinarily observed in urban environments have revealed no irreversible toxic response in the respiratory tract. In contrast, however, lifetime intermittent exposure of rats to benzo[*a*]pyrene (BP), a known carcinogen, in conjunction with SO_2 suggests that SO_2 may be acting as a cocarcinogen (Laskin, Kuschner, Sellakumar & Katz, 1976). The data from this study are summarized in Table 1. Although the significance of the temporal aspect of SO_2 exposure relative to that of BP is difficult to assess, consideration of the data *in toto* strongly suggests a role for SO_2 in the aetiology of these bronchogenic squamous-cell carcinomas.

Discussion

Although *in vitro* research has been extremely useful in increasing our knowledge of sulphite chemistry and of the potentially toxic reactions of sulphite, *in vitro* systems have, in general, been poor models for predicting mammalian toxicity, largely because *in vivo* mammalian sulphite concentrations have been greatly overestimated. Extrapolations of *in vitro* data to the *in vivo* situation have often been erroneous (Inouye *et al.* 1978; Kaplan *et al.* 1975; Schneider & Calkins, 1970), primarily because of ignorance of sulphite metabolism. Now, however, quantitative data relating exogenous sulphite exposure to *in vivo* sulphite concentrations in mammals are available, and provide a basis for evaluation of the *in vivo* implications of *in vitro* data. Thus, whereas plasma-sulphite concentrations of up to approximately 100 μM were observed in a rhesus monkey ingesting 2 mmol sulphite/kg/day, most of the sulphite toxicity demonstrated *in vitro* resulted from concentrations that were considerably higher.

Further, because of its rapid metabolic clearance by sulphite oxidase, chronically ingested sulphite does not accumulate in the tissues and reach an elevated steady-state concentration but is rapidly eliminated after absorption, giving sporadic brief episodes of elevated tissue-sulphite levels; these will not sustain sulphite adducts which are unstable in the absence of free sulphite. Therefore, the toxicological significance of the reversible reactions of sulphite described in the earlier sections is probably slight or nil under conditions of intermittent exposure of body tissues to sulphite—the expected pattern of human exposure.

Compared to reversible reactions, the irreversible reactions of sulphite are of potentially greater toxicological significance. The sulphitolysis of thiamine, for example, although a relatively slow reaction, results in significant destruction of the vitamin in stored diets as well as in the gut.

The sulphite-catalysed deamination of cytosine and 5-methylcytosine residues in DNA is an irreversible reaction of possible toxicological significance. Shapiro (1977) has calculated, on the basis of the *in vitro* rate of conversion of cytosine to uracil under physiological conditions, that a sulphite concentration of only 0·3 μM is required to double the spontaneous mutation rate in humans. The toxicological implications of this calculation are now unclear, however, in the light of the apparent lack of involvement of cytosine residues in C:G to A:T transitions. As pointed out earlier, this is an area of current investigation.

Another group of reactions with toxic potential are the reactions of free radicals, produced by the autoxidation of sulphite, with tryptophan, methionine (and certain other sulphides), DNA and olefins. Again, reactions with the first three substrates are apparently

Table 1. *Squamous-cell carcinomas in rats chronically exposed to benzo*[a]*pyrene and* SO_2*, alone and in combination**

Exposure conditions (on 5 days/wk)	No. of rats		Incidence (%)
	Per group	With carcinomas	
Filtered air	15	0	0
10 ppm SO_2 (6 hr)	15	0	0
BP† (1 hr)	30	1	3
10 ppm SO_2 (6 hr) followed by BP† (1 hr)	30	2	7
BP† with 4 ppm SO_2 (1 hr)	45	4	9
10 ppm SO_2 (6 hr) followed by BP† with 4 ppm SO_2 (1 hr)	46	9	20

*Data from Laskin *et al.* 1976.
†Exposure to benzo[*a*]pyrene at 10 mg/m^3.

irreversible, while information on the reversibility of the latter is not available. The implications of these reactions include damage of membranes due to free-radical attack on their unsaturated lipids, and chromosome aberrations resulting from sulphite-induced DNA chain breaks. Although free-radical reactions proceed at lower concentrations than many of the ionic reactions of sulphite, it is difficult to speculate on their occurrence *in vivo* because of the presence of numerous free-radical scavengers which prevent significant sulphite autoxidation, even at favourable sulphite concentrations, and because superoxide dismutase may inhibit the initiation of autoxidation by O_2^-.

Most of our knowledge of mammalian sulphite toxicity originates from experiments in which sulphite oxidase-competent rats were fed large quantities of sulphite. From these data most toxicologists would legitimately conclude that the hazard to humans of sulphite consumption at present levels is very low. However, the role of sulphite-oxidase activity in sulphite toxicity has not as yet been adequately investigated. We have pointed out previously that, with respect to sulphite-oxidase capacity, the rat is a poor model for humans. There are, in addition, essentially no data on the variability of sulphite-oxidase activity in the human population. Further, little is known of the quantitative relationships between intake of sulphur-containing amino acids, sulphite-oxidase activity and endogenous sulphite concentration.

Given the relatively low mean sulphite-oxidase capacity of humans, the possibility of significant genetic variation in sulphite-oxidase activity among individuals, the uncharacterized variable of dietary sulphur-amino acid content and the generally low human consumption of sulphites, it appears that the importance of endogenous sulphite generation relative to exogenous sulphite intake may have been underestimated in most previous investigations of sulphite toxicity. Further, endogenous sulphite is produced intracellularly in relatively close proximity to DNA and other potential target molecules, while ingested sulphite must traverse several barriers before reaching these sites.

In human sulphite oxidase-deficiency disease as well as in sulphite oxidase-deficient rats, steady-state sulphite concentrations in the micromolar range have resulted from purely endogenous sources. Under these conditions, reversible reactions of sulphite can be of physiological significance provided the sulphite adduct is reasonably stable. As an example, it was previously stated that sulphite reacts readily, although reversibly, with NAD when associated with LDH, forming an enzymatically inactive adduct with a pH-independent dissociation constant of approximately 10^{-7} M (Parker *et al.* 1978). Using this K_d, one can calculate that in the presence of 50 μM $SO_3^{=-}$, more than 99% of the NAD bound to LDH would be in the form of the sulphite adduct and, therefore, presumably inactive. There is some discrepancy, however, between this calculated value and the experimentally determined inhibition of LDH *in vitro*. Ciaccio (1966) and Oshino & Chance (1975) demonstrated 50% inhibition of the enzyme by 350 and 200 μM sulphite, respectively. Similar data for other enzyme–cofactor–sulphite adducts and other types of sulphite addition products suggest that metabolic disturbances may result from steady-state sulphite concentrations in the micromolar range, as can be expected to occur in animals sufficiently deficient in sulphite oxidase.

A series of enzymes that are inhibited by micromolar concentrations of sulphite are the sulphatases, most notably sulphatase A. Cerebroside sulphatase, which is thought to be identical to sulphatase A, is deficient in cases of metachromatic leucodystrophy (MLD), a genetically determined disease in which myelin degeneration is associated with accumulation of cerebroside sulphate (Moser, 1972). There is also a series of genetically determined diseases (mucopolysaccharidoses) many of which are caused by, or associated with, functional deficiencies of one or more other sulphatase enzymes (Roy, 1976). Nearly all of these diseases are characterized by mental retardation as well as by physical defects, especially those of bone. The possibility of inhibition of sulphatase enzymes *in vivo* by sulphite was recognized by the investigators who first identified sulphite oxidase-deficiency disease. Analysis of the urine and tissues from a patient who died of this disease revealed no significant increase in tissue or urinary sulphate esters as would be expected if sulphatases had been inhibited (Percy, Mudd, Irreverre & Laster, 1968). However, these results are difficult to interpret since the severely decreased availability of sulphate in this patient would have resulted in a reduced rate of sulphate ester synthesis.

Several experiments reviewed in this paper have suggested, or are consistent with, a role for sulphite as a cocarcinogen. Certainly the development of bronchogenic squamous-cell carcinomas following inhalation exposures to BP and SO_2 (Laskin *et al.* 1976) and the *in vitro* demonstration of sulphite enhancement of UV mutagenicity (Mallon & Rossman, 1981) support this hypothesis. In addition, the appearance of mammary adenocarcinomas in young sulphite oxidase-deficient rats might result from an interaction of sulphite and tungsten. Although this is admittedly speculative, the cocarcinogenesis hypothesis should continue to be investigated.

The *in vitro* investigations of sulphite reactivity and toxicity reviewed in this paper have suggested an array of mechanisms that could produce toxicity in mammals. Consideration of this information and the data from *in vivo* evaluations of sulphite toxicity in the light of mammalian sulphite metabolism, makes possible the elimination of some of these mechanisms as unlikely and the selection of others as being of greater potential and as deserving further investigation.

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FORTHCOMING PAPERS

It is hoped to publish the following research papers and other communications in the next issue of *Food and Cosmetics Toxicology*:

Intestinal absorption of two polymeric food dyes in man. By P. D. Walson, D. E. Carter, B. A. Ryerson, D. Clark and T. M. Parkinson.

Acute toxicity of thioguaiacol and of Versalide in rodents. By K. R. Butterworth and P. L. Mason.

Long-term toxicity study of ethyl methylphenylglycidate (strawberry aldehyde) in the rat. By D. Dunnington, K. R. Butterworth, I. F. Gaunt, P. L. Mason, J. G. Evans and S. D. Gangolli.

Inhibition of phylloquinone epoxide-dependent carboxylation of microsomal proteins from rat liver by 2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone. By O. Takahashi and K. Hiraga.

Effects of dietary bran and the colon carcinogen 1,2-dimethylhydrazine on faecal β-glucuronidase activity in mice. By J. F. London, N. K. Clapp and M. A. Henke.

Effets des fongicides, nabame et zinèbe, sur l'activité des oxygenase et sur la teneur en thiolates dans les microsomes hépatiques du rat. By N. Miladi, S. Attéba, E. Dooh-Priso, C. Desfontaines and R. Albrecht.

A 90-day toxicity study of phenylethyl alcohol in the rat. By E. Owston, R. Lough and D. L. Opdyke.

Effect of indole on *N*-nitrosodimethylamine demethylase in rats treated with carbon tetrachloride. By M. H. Mostafa, M. Ruchirawat and E. K. Weisburger.

Citrinin mycotoxicosis in broiler chickens. By N. A. Q. Mehdi, W. W. Carlton and J. Tuite.

The pharmacokinetic profiles of ochratoxin A in pigs, rabbits and chickens. By P. Galtier, M. Alvinerie and J. L. Charpenteau.

Aflatoxins B_1 and M_1: Tissue residues and feed withdrawal profiles in young growing pigs. By G. L. Neff and G. T. Edds.

Mutagenicity of commercial hair dyes in *Salmonella typhimurium* TA98. By G. Albano, A. Carere, R. Crebelli and R. Zito.

In vivo skin penetration studies of 2,4-toluenediamine, 2,4-diaminoanisole, 2-nitro-*p*-phenylenediamine, *p*-dioxane and *N*-nitrosodiethanolamine in cosmetics. By F. N. Marzulli, D. M. Anjo and H. I. Maibach.

The effects of nitrilotriacetate on cation disposition and urinary tract toxicity. By R. L. Anderson, C. L. Alden and J. A. Merski.

Mutagenicity of Maillard browning products obtained from a starch/glycine model system. By C.-I. Wei, K. Kitamura and T. Shibamoto. (Short Paper)

Volatile nitrosamines: Analysis in breast fluid and blood of non-lactating women. By A. A. Melikian. E. J. LaVoie, D. Hoffmann and E. L. Wynder. (Short Paper)

A prospective analysis of the potential risk associated with inhalation of aflatoxin-contaminated grain dusts. By C. S. Baxter, H. E. Wey and W. R. Burg. (Review Paper)

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The Journal publishes original papers and reviews relating to the interests of the British Industrial Biological Research Association. This is a wide-ranging field covering all aspects of toxicology but with particular reference to food and cosmetics. The Journal aims to be informative to all who generate or make use of toxicological data.

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