Methods For Assessing the Effects of Mixtures of Chemicals
Edited by V. B. Vouk, G. C. Butler, A. C. Upton, D. V. Parke and S. C. Asher

4 *Metabolism and Biochemical Mechanisms of Combined Action**

4.1 INTRODUCTION

This section considers some basic biochemical mechanisms that may be involved in the interaction of chemicals. The manner in which this knowledge can be used to develop methods for the study of chemical interactions is explored and priorities are suggested. This section is divided into two major parts. The first part discusses the basic mechanisms of chemical interactions at the level of cells and organisms, and the factors that modulate the effects of mixtures on populations. The second part discusses methods for studying these interactions and is divided into basic approaches, methods used during the chemokinetic phase, and methods used during the chemodynamic phase of chemical metabolism.

There are only a limited number of instances in which the biochemistry of chemical interactions at the cellular level is known. Nevertheless these examples indicate the approach to be followed. Some of the best established cases are listed below.

(1) Several heavy beer drinkers, imbibing approximately 10 litres a day, died of cardiomyopathy caused by the toxicity of a cobalt-containing additive, potentiated by malnutrition. The cobalt intake was only 1/30 of the therapeutic dose but the lack of some amino acids in the drinkers' diet enabled the cobalt to complex with an α-lipoic acid thereby inhibiting some metabolic systems, and ultimately depriving the heart muscle of oxygen (Grice *et al.*, 1981).

(2) In Itai-Itai disease the effect of chronic exposure to cadmium is potentiated by nutritional deficiency and endocrine imbalance. Painful bone changes were observed when elderly women, apparently deficient in vitamin D and calcium, were exposed to high levels of cadmium through contaminated local staple food (Hamagami *et al.*, 1978).

(3) A classical instance of synergism between two compounds is the effect of *O*-ethyl-(*O*-4-nitrophenyl)phenylphosphonothioate (EPN) on the insecticidal

* This section was prepared by a working group chaired by E. Somers. The members were J. W. Bridges (Rapporteur), B. Ketterer, L. J. King, F. Matsumura, D. B. McGregor, P. N. Newberne, J. Parizek, D. B. Peakall, and A. R. Wellburn.

action of malathion (DuBois, 1961). EPN is an extremely effective inhibitor of carboxylesterases which are known to be the major detoxication enzymes for malathion. EPN irreversibly inhibits tissue carboxylesterase by phosphorylation of the active site.

4.2 BASIC MECHANISMS OF INTERACTION

Interactions between chemicals may occur in the pre-absorption (exposure), chemokinetic (transfer) or chemodynamic (action) phases of the toxication process (Ariens, 1972). Chemical interactions are particularly likely if the exposure levels are high, the chemicals involved are reactive or readily metabolized to more toxic products, the points of interaction with biological systems are readily saturable, and alternative non-essential binding sites are unavailable. Interactions may be different for acute and chronic exposure conditions because of various biochemical mechanisms such as repair processes or induction of detoxifying or activating enzymes.

In pharmacology the combined action of several chemicals can be considered within two categories depending on the type of influence of one chemical on the action of the other: non-interactive (independent and similar joint action), and interactive (synergistic or antagonistic).

4.2.1 Interactions at the Cellular Level

4.2.1.1 Interaction in the Pre-absorption (Exposure) Phase

The components of a mixture may interact either chemically or physically in such a way that the chemical composition of the mixture and/or the accessibility of its components to the interior of the cell are altered. Chemical reaction can result in new chemical species, such as the formation of nitrosamines from nitrite and secondary amines, the association of ionic species of opposite charge to form lipid-soluble ion pairs, or the chelation of metal ions by organic ligands to produce absorbable lipophilic complexes. Small molecules, both organic and inorganic, may be adsorbed on the surface of particulate matter (e.g. fibres such as asbestos and dusts composed of silicates), thus altering their contact with the cell membrane. In addition, the presence of extracellular enzymes and microorganisms in the environment of the cell can cause changes in the chemical composition of a mixture of compounds.

The pH of the environment in immediate contact with the cell membrane may influence the rate and the extent to which reactions and interactions take place, and will affect the degree of ionization of any weak acid or base that is present. Changes in the pH of the extracellular fluid may, therefore, alter the rate of absorption and excretion of chemicals.

4.2.1.2 Interactions in the Chemokinetic Phase

Cellular Uptake and/or Excretion Cellular uptake and excretion of a chemical may involve one or more of the following basic mechanisms: passive diffusion, active and facilitated transport, and endo- and exocytosis. Passive diffusion, which occurs in all cell types, is the most common mechanism of uptake and excretion of lipophilic chemicals, but is unlikely to be a common process in interactions except when the cell membrane is damaged by one agent and the rates of transfer of the chemical and/or its metabolites are affected, or when pH or protein binding changes are brought about in the extracellular fluid by the interacting agent. It should be noted that the presence of a polar cell wall, found in plants and bacteria, may considerably restrict the extent of passive diffusion.

Facilitated and active transport, which is potentially a very important location of chemical interactions, is a relatively uncommon mechanism of cellular uptake for exogenous chemicals, except for compounds that have a close structural relationship to endogenous compounds; however, it is important for the uptake of several inorganic ions. Both passive and active transport have an important role in the excretion of many organic anions and cations including the common metabolites of exogenous chemicals such as glucuronides. Many examples of competition between exogenous chemicals for active excretion are known, particularly in renal tubular cells.

Endo- and exocytosis are important mechanisms of the transmembrane transport of macromolecules, hormones, and growth factors in some cell types. Information on the effect of one chemical on the endo- or exocytosis of another compound is poorly documented; one example, however, is the effect of the tumour promoter 12-*O*-tetradecanoylphorbol-13-acetate on the transport of an epidermal growth factor (Ivanovic and Weinstein, 1981). It may be expected that agents which cause significant damage to cell membranes will inhibit these mechanisms.

Cellular Distribution After uptake into a cell, a chemical may remain unchanged in solution, it may be deposited, or it may become reversibly or irreversibly bound to endogenous cellular constituents of low or high molecular weight. Relatively little is known about the factors that influence the partitioning of a chemical between the various cell compartments (organelles) although it appears that lipophilicity favours accumulation within the endoplasmic reticulum and that several carboxylic acids and inorganic ions are actively taken up by, for example, mitochondria.

The point of active transport across such organelle envelopes is a likely location of chemical interactions. Several cellular proteins, such as ligandin, offer more or less specific binding sites for exogenous chemicals, and these are also likely sites of competition. Surprisingly, there are relatively few documented examples of interactions of this type involving cellular proteins despite the

repeated demonstration of the importance of competition between chemicals such as albumin for extracellular protein-binding sites.

Whether or not adverse consequences arise depends on the extent to which the displaced chemical is able to adopt sites on other non-essential cellular components. Reaction with a cellular component such as glutathione may result in a rapid clearance of the complex from the cell, while the build-up of particular inorganic ions, for example Cd^{2+}, Hg^{2+}, or Cu^{2+} (alone or in combination), can trigger in certain cells the synthesis of specific binding proteins, such as metallothionein. These proteins have a very high affinity both for the ion itself and for similar ions, thereby favouring the retention of these ions within the cell.

Biotransformation For lipophilic chemicals, biotransformation is often an essential prerequisite for successful excretion. Within different types of cells and between individuals within a species, there are wide qualitative and quantitative variations in the enzymes that are capable of metabolizing exogenous chemicals. The metabolic reactions between exogenous chemicals and enzymes that occur on exposure to a mixture of compounds may differ, depending on whether the immediate effect of a single exposure is considered, or whether the metabolic response is evaluated after repeated exposures, after which time the cells may have adapted to the chemicals. Although many cellular enzymes are involved in the metabolism of exogenous compounds, the cytochrome P-450 mixed function oxidases appear to be particularly likely sites of biotransformation because of their broad substrate specificity and the fact that the rate of metabolism by these enzymes often limits the rate of removal of the chemical from the cell.

Interaction between exogenous chemicals may occur by saturation of the active site of a particular enzyme by one or more substrates. This may cause redistribution of one chemical to other enzymes, resulting in the expression of previously underused pathways of biotransformation. For some enzymes, this redistribution may lead to the activation of latent enzymes. In other instances, insufficiency of essential cofactors within the cell for the preferred reaction may result in the involvement of other enzymes with no or with different cofactor requirements.

The persistence of inhibitory effects depends on the turnover rate of the enzyme and/or the rates of degradation and synthesis of the enzyme itself. Commonly, the cell responds within hours to a persistent inhibition of the microsomal mixed function oxidases by induction of new enzyme proteins, frequently of a different isoenzymic form from that present initially and often more suited to the metabolism of the inhibitory agent. These induced isoenzymes may metabolize the inhibitory agent and other compounds in the mixture through different metabolic pathways from those of the isoenzymes of the uninduced cell. Thus, the metabolic interactions observed in a cell after repeated exposure may differ considerably from those seen immediately after exposure because of the alteration in the enzyme complement.

When considering metabolic interactions in particular, it must be remembered that the response of the cell depends on both the type of tissue and the species concerned. Metabolic fate in microorganisms and plants may be quite different from that in vertebrates.

4.2.1.3 Interactions in the Chemodynamic Phase

When identifying mechanisms of toxic action it is worth bearing in mind that the biological reaction under observation may not be the primary site at which the toxifying process is initiated. Cell injury may be initiated by the formation of reversible or irreversible complexes of the chemical and/or its metabolites with a variety of cell components (such as enzymes, nucleic acids, membrane proteins, receptor sites and cofactors) or by physicochemical changes (e.g. changes in pH, redox potential, ionic composition, solubility or pO_2) within the cell or its immediate environs. Recently, attention has been focused on the role of reactive species in the toxifying process, namely:

(1) the formation of highly reactive electrophilic or free radical metabolites by the enzymes that metabolize exogenous chemicals; and
(2) the initiation of the production of active oxygen species, such as superoxides, hydroxyl radicals, and hydrogen peroxide.

The Genome Many carcinogens are able, directly or by means of reactive metabolites, to form adducts with DNA which may cause errors in DNA transcription. Several mechanisms have been proposed whereby mutations could be modulated by the presence of a second chemical. However, in most cases where interactions between chemicals have been observed, the mechanism involved has not been characterized nor has its *in vivo* relevance been established. For example, benzo[*e*]pyrene enhances the mutagenic response to benzo[*a*]pyrene in *Salmonella typhimurium* TA98 while the mutagenesis of amino acid pyrolysis products and of aflatoxin B can be inhibited *in vitro* by haemin or unsaturated fatty acids. Caffeine has also been shown *in vitro* to exacerbate the effect of mutagenic chemicals by inhibiting DNA repair mechanisms (McGregor, this volume).

Enzyme Inhibition Many chemicals exert their primary toxic action by competitive, non-competitive reversible, or non-competitive irreversible inhibition of enzymes that have important roles in the normal functioning of the cell; for example, dinitrophenol causes the uncoupling of oxidative phosphorylation in the mitochondria. Chemical interactions involving enzyme inhibition would normally be anticipated to cause additive effects unless the inhibition by the chemicals occurs at sequential stages of a key metabolic pathway, in which case a synergistic action might ensue.

Cofactors Chemically mediated increases in the ratios of ADP/ATP or of oxidized cofactor/reduced cofactor, if sustained, will result in toxic action. Depletion of reduced cofactors may occur by direct reaction with a chemical and/or its metabolites, by futile cycling, or by inhibition of cofactor synthesis. Depression of (reduced) glutathione (GSH) may enable reactive metabolites to improve their targeting to cell constituents. Thus the electrophile 1,3-*bis*(2-chloroethyl)-1-nitrosourea reacts specifically with GSH reductase causing its inactivation, and thereby preventing the regeneration of GSH from the oxidized form of glutathione (GSSG). When administered with other drugs that decrease GSH levels directly, such as diethyl maleate, additive effects on the detoxication systems that are dependent on GSH may occur (Babson and Reed, 1978).

Cell-surface Receptor Sites Tumour promoters such as phorbol esters and teleocidin bind to specific high-affinity receptors on the surface of mouse fibroblasts in culture and at the same time cause inhibition of the binding of the epidermal growth factor (EGF) to its cell-surface receptors (Ivanovic and Weinstein, 1981). Benzo[*a*]pyrene and other polycyclic aromatic hydrocarbons have similar effects but apparently through action dependent on cytoplasmic and/or nuclear events. In contrast, corticoids increase EGF receptor binding. Thus the combination of corticoids with tumour promoters or benzo[*a*]pyrene causes opposite effects with respect to EGF binding (Ivanovic and Weinstein, 1981).

Chemicals Selective for a Specific Cell Compartment Diethyl maleate cannot penetrate mitochondria and therefore has access only to cytosolic GSH. Consequently, the GSH depletion it brings about only enhances the lipid peroxidation resulting from the relatively low oxygen utilization of the endoplasmic reticulum. In contrast, ethacrynic acid can also enter the mitochondrion, where most oxygen is consumed, and GSH is in high demand. GSH depletion by ethacrynic acid greatly enhances lipid peroxidation in the mitochondria and, when combined with the effects of diethyl maleate in the cytosol, is highly cytotoxic (Meredith and Reed, 1982).

Sequential Action of Chemicals Several of the effects mentioned above may occur in sequence when a cell is exposed to a chemical mixture. An example is the effect of a tumour initiator acting on the genome followed by a subsequent action of a tumour promoter.

4.2.2 Interactions Involving Intact Organisms

4.2.2.1 Microorganisms

Microorganisms in soils and aquatic sediments and those associated with animals and plants play a major role in the metabolism of nutrients and many

exogenous chemicals. Microbial biotransformation may involve incidental metabolism (i.e. involving no net gain of energy), catabolism (i.e. energy-yielding biotransformation) or detoxication. Since most environmental chemicals are present at low concentrations and are structurally dissimilar from naturally occurring substances, the bulk of microbial metabolism probably proceeds via incidental metabolism. Major metabolic reactions are oxidation, hydrolysis, and reduction. (Note that gut microflora oxidation reactions are uncommon because of the highly anaerobic conditions that prevail in the gut.)

A common type of interaction is one in which the profile of microbial metabolism is so drastically affected by some components of the mixture that the entire metabolic pattern of other chemicals is changed. For instance, if the mixture contains bactericidal or fungicidal components, the total metabolic activity may be significantly reduced. Examples are chlorinated phenols, antibiotics, and products containing organomercury. Mixtures containing nutritional components may stimulate microbial growth. For instance, pesticides discharged with a large quantity of nutrients (e.g. pesticide residues in composts) may show enhanced degradation rates. Microbial populations may also be affected by pH and changes in their microenvironments. For example, under acidic conditions fungi could predominate and at neutral pH bacteria may thrive, while a shift from a predominantly aerobic environment to an anaerobic environment could result in an entirely different population of microorganisms (Brock, 1970).

A more subtle type of reaction may occur at the subcellular level. For instance, many organophosphate and carbamate insecticides are potent antiesterase agents. The presence of such compounds in a chemical mixture could prevent hydrolytic degradation of the entire mixture. Another type of interaction can occur with oxidative reactions. Since many microbial oxidative systems such as monooxygenases involve iron–sulphur proteins (unlike the corresponding enzyme systems in higher animals), they are extremely susceptible to all forms of —SH inhibitors, such as organomercury compounds.

4.2.2.2 Plants

Plants form the largest living biomass in all living systems. They have a wide range of secondary metabolic processes and three distinct genetic pools, in addition to possessing most of the biochemical pathways associated with vertebrates. They are particularly vulnerable to both wet and dry deposition of primary atmospheric pollutants such as sulphur dioxide or nitrogen dioxide and to their products in solution, as well as to the secondary pollutants such as ozone or peroxyacetylnitrate. These pollutants cause depression both of growth and of net photosynthesis. The overall toxic action of these pollutants may be additive, synergistic, or antagonistic (Ormrod, 1982). Man-made organic chemicals may have intentional or unintentional effects on plants and plant growth regulation

(e.g. herbicides) and are widely used. However, in terms of significant interactions, there appear to be too few clear indications of phytotoxicity of exogenous chemicals as demonstrated in the synergistic action of carbaryl (and other organophosphate and carbamate insecticides) and propanil (rice herbicide) and the antagonistic action of protectants (e.g. naphthylic anhydride) and *S*-ethyldipropylcarbamothioate herbicides.

Inorganic atmospheric pollutants, however, have been shown to display potent synergistic action. Possible biochemical explanations for the significant depression of growth caused by the interactions between sulphur dioxide and nitrogen dioxide include membrane and enzyme effects (Ormrod, 1982). In the chloroplast, when sulphur dioxide and nitrogen dioxide are dissolved in water of high pH the two products are sulfite and nitrite. Individually these pollutants, which occur normally at very low concentration, are reduced by the photosynthetic electron transport chain and then used for the synthesis of proteins. However, under conditions of sulphur dioxide + nitrogen dioxide fumigation, the concentration of these anions may increase and cause the formation of free radicals. These radicals may produce effects similar to those produced by ozone, namely a deterioration in the ability of photosynthetic membranes to maintain an effective proton gradient across the membrane (Robinson and Wellburn, this volume). This in turn reduces the ability of bioenergetic membranes to generate ATP efficiently and this is reflected ultimately in synergistic depressions of plant growth (Koziol and Whatley, 1984).

The use by the plant of normal metabolic pathways for detoxication (e.g. transformation of nitrites to amino groups of proteins) is also hindered by chemical interactions. The inducible enzyme nitrite reductase normally converts nitrite to ammonia which is then incorporated into glutamate. This induction takes place in most species after nitrogen dioxide fumigation but is unaffected by sulphur dioxide treatment. However, in the mixed treatment (sulphur dioxide + nitrogen dioxide), sulphur dioxide completely inhibits the inducibility of nitrite reductase thereby removing the ability to detoxify additional nitrite (Wellburn *et al.*, 1981). It is not known at what level this interaction occurs but it is presumably at the level of genome expression.

4.2.2.3 Animals

Absorption Interactions at absorption sites in the whole animal may depend on the differences in cell types at the point of entry (e.g. the presence of a relatively impermeable layer such as the skin, or the presence or absence of specific carrier molecules). Dimethyl sulphoxide has been shown to enhance the absorption of lipophilic compounds through the skin, and hydrated skin is more permeable to both lipophilic and hydrophilic compounds. The presence of gut microflora in contact with the intestinal mucosa permits several physical and metabolic interactions which may influence the absorption of components from a mixture.

The presence of cadmium ions in the gut may impair the absorption of iron, zinc, and calcium, possibly by interaction with the specific carriers for these essential metals.

Distribution Differences in the distribution of chemicals among tissues may occur, for instance, as a result of displacement of a compound from its extracellular binding on albumin by another compound. This effect is well documented for drug interactions; for example, the displacement of bilirubin by bacteriostatic sulphonamides in neonates may cause abnormal deposition of bilirubin in tissues such as the brain, leading to kernicterus.

Biotransformation In animals, the biotransformation enzymes tend to be most concentrated in those organs that are more exposed to the environment (skin, lung, intestine) and in the excretory organs (liver and kidney). Overall, the liver is the most active organ in the metabolism of exogenous chemicals. The tissue distribution of these biotransformation enzymes is of particular interest in relation to the toxicity of target organs. Many enzymes that metabolize exogenous chemicals exist in several isoenzymic forms (with different substrate, inhibitor, and inducer specifications) and the distribution of these forms varies greatly among tissues and species. This may result in different tissues and different species showing marked variation in the extent of interactions brought about by particular chemical combinations. The total activity of a class of biotransformation reactions, such as oxidations, also varies widely with species. The level of hepatic activity of mixed function oxidases (MFOs,), epoxide hydrolases, and glucuronyl transferases increases in the following order: fish < birds < mammals (Walker, 1980). In general, man has lower MFOs per gram of liver than laboratory animals. These species differences may be reflected in differences in bioaccumulation and toxicity. For example, differential retention of the more highly chlorinated polychlorinated biphenyls (PCBs) occurs in several species due to the ability of these species to metabolize and thereby excrete PCBs with a lower chlorine content. In fish with limited metabolic transforming capacity, the composition of the PCB mixture in the tissues tends to be similar to the composition of the mixture to which they are exposed.

Retained PCBs may also cause induction of some isoenzymes of cytochrome P-450. Induction of MFOs may enhance the metabolism of other chemicals which could result in reduced toxicity or, if a pathway for the formation of a reactive metabolite is induced, increased toxicity. Selective inhibitors of MFOs (pesticide synergists such as sesamex (Metcalf, 1967), furans, or imidazoles such as cimetidine) may have the opposite effect.

Excretion The excretion of exogenous chemicals and their metabolites occurs principally via urine or bile. The significance of these routes shows marked interspecies variation and this may influence the degree of chemical interaction.

The principal interaction is that of competition for the active secretion processes, principally the secretion of organic anions. The retention of one or more of the competing chemical species increases both their biological half-time and their concentration in the excretory organs and other tissues, and therefore the expression of adverse effects is possible at sites distant from the point of excretion. Use has been made of this phenomenon in drug therapy; for example, probenecid causes retention of penicillins and thereby enhances their bactericidal activity. The possibility of similar interactions in the biliary secretion process can also be anticipated.

Chemodynamics Apart from direct interaction at receptor sites, for example by agonists and antagonists, the possibility of interactions arising from the action of an exogenous chemical on one cell type that may influence the response of another cell type must also be considered. Such interactions may be expected where the primary target cell secretes a hormone which modifies the physiological and metabolic competence of other tissues. Hormonal modification of the biotransformation capacity of exogenous chemicals is a recognized phenomenon, and interference with this phenomenon by some chemicals may have a considerable influence on the ability of the animal to metabolize other absorbed compounds.

Viral Infections Viral infections, even subclinical, may also affect the toxicity of exogenous chemicals in various ways and by different mechanisms. For example, viral hepatitis in human subjects may lead to impairment of the liver MFOs and conjugation enzymes, resulting in increased plasma half-times, lower clearances, and the increased pharmacological activity or toxicity of several drugs and chemicals. Recovery of the intermediary metabolic function may be much slower than recovery of the normal liver function, thus affecting interactions between exogenous chemicals. Similarly, viral infections in animals may potentiate chemical carcinogenicity, with both the virus and the chemical acting simultaneously or sequentially at genetic or epigenetic levels (Galloway and McDougall, 1983; Schwab *et al.*, 1983). The acquisition of some viral genes may be associated with cell transformation. Cellular oncogenes may mediate oncogenesis subsequent to initiation and might be derepressed by insertion of viral DNA or by gene translocations following chemically induced mutations.

4.2.3 Factors that Modulate the Effects of Chemical Mixtures on Populations

Factors that contribute to the risk of disease in animal and human populations can be grouped for convenience into two major categories: those that arise internally and those that are derived from external sources (Table 4.1). Increased risk associated with intrinsic factors is of concern but the extrinsic factors,

Table 4.1 Factors contributing to risk of disease in human populations

A. *Intrinsic factors*
1. Dietary deficiencies (internal)
2. Metabolic deficiencies
3. Hormonal factors
4. Internal chemicals
B. *Extrinsic factors*
1. Life-style
(a) alcohol
(b) tobacco
(c) diet (food and water)
(d) drugs
2. Climate and air pollution
3. Exposures in the workplace

particularly life-style, are of utmost significance, especially if exposure to complex mixtures is involved.

4.2.3.1 Intrinsic Factors

Internal or intrinsic factors that contribute to the risk of disease are listed in Table 4.1. Dietary deficiencies can arise, for example, from an internal deficit of vitamin B_{12} caused by a lack of some gastric factors, resulting in failure to absorb the vitamin. Metabolic deficiencies can be represented by a congenital defect where an enzyme such as phenylalanine hydroxylase is missing or in short supply, and so phenylketonuria and its sequelae ensue. Hormonal factors include obesity with synthesis of excessive amounts of hormones, or deficiency of thyroxine occasioned by consumption of certain cyanogenic materials, which in turn inhibit the formation of the hormone. The feedback results in thyroid hyperplasia.

Numerous examples can be given of instances where endogenous chemicals contribute to risk. Alterations in the microflora that flourish in the bowel to act on fats and bile acids could be related to the incidence of large-bowel cancer. Formation of *N*-nitroso compounds from nitrite and amines in the body is of particular concern in gastric and bladder carcinogenesis (Doll and Peto, 1981; NAS/NRC, 1982; Newberne, this volume). Whole animals are thus exposed to real or putative toxins, carcinogens, or conditions which derive from internal reactions or other alterations.

4.2.3.2 Extrinsic Factors

The first major extrinsic factor that may modulate the response to a chemical can be considered under the general term life-style, and includes a range of social,

geographic, cultural, and religious factors and conditions (Table 4.1).

Ingestion of alcohol has a marked effect on the metabolism of other exogenous chemicals present in the body, whether administered drugs or adventitious environmental chemicals, the nature of the effect being largely a function of the duration of exposure. Acute exposure to ethanol may inhibit cytochrome P-450 dependent MFOs, which are effective in metabolizing exogenous chemicals, or it may disturb the lipid bilayer membrane (Hoyumpa and Schenker, 1982). Similarly, ethanol potentiates the toxicity of halogenated hydrocarbon solvents. Repeated exposure to ethanol, on the other hand, may selectively increase the activity of enzymes that metabolize exogenous chemicals so that increased elimination of drugs such as pentobarbital or meprobamate is achieved (Hoyumpa and Schenker, 1982).

Epidemiological studies of asbestos workers (McDonald, 1980) and miners exposed to radon daughters (Damber and Larsson, 1982) have clearly established that cigarette smoking potentiates the adverse health effects of these agents. The polycyclic aromatic hydrocarbons present in cigarette smoke are potent inducers of microsomal enzyme activity; hence cigarette smoke can accelerate the biotransformation *in vivo* of such drugs as phenacetin, antipyrine or caffeine (Conney, 1982). Simple chemical antagonism resulting from the chemicals in smoke may be responsible for reduced serum ascorbic acid levels in smokers (Pelletier, 1975).

Diet appears to be a major factor in causing cancer and in modulating the response to chemicals. Chemical interactions play an important role in exposure to mixtures that ultimately results in cancer or other forms of chronic disease. The postulate that nitrite, derived from reduction of nitrate and naturally occurring amines in foods, can react to form carcinogenic nitrosamines in the gastrointestinal tract is substantiated by numerous studies (Mirvish, 1981; Ohshima and Bartsch, 1981; Tannenbaum *et al.*, 1978). Ascorbic acid and vitamin E protect against such reactions *in vivo* and *in vitro* (Ivankovic *et al.*, 1975; Mirvish, 1981). Selenium, toxic at high concentrations, has a protective role against mercury, cadmium, and thallium toxicity (NAS/NRC, 1980). Selenium also protects against carcinogenesis in the liver caused by aflatoxin B, where it diminishes the activity of the enzymes that produce reactive metabolites from aflatoxin B. The same is true for other carcinogens and target organs, including 7,12-dimethylbenzo[*a*]anthracene and mammary gland cancer, and dimethylhydrazine and colon cancer (NAS/NRC, 1982).

Retinoids are another class of nutrients involved in modulating the response of mammalian species to chemicals. This generic term includes all materials with vitamin A activity and their synthetic analogues, many of which are effective anticarcinogens in animal models (Sporn and Newton, 1981). Vitamin A deficiency is associated with cancer of the lung, larynx, and urinary bladder in human populations and with cancer of several epithelial structures in experimental animals (NAS/NRC, 1982). In the United States, the National Cancer

Institute now supports 17 intervention programmes with chemopreventive measures (Greenwald, 1984). One of these is a prospective study using β-carotene in 25 000 physicians and another is a pilot study with selenium in a population of dentists. These examples of nutrient effects point the way towards research needed in efforts to evaluate chemical mixtures in the environment.

Drugs are not taken in isolation. Their activity can be affected by many environmental chemicals such as pesticides and polycyclic aromatic hydrocarbons that induce liver microsomal enzymes. Workers exposed to DDT and lindane metabolize antipyrine twice as rapidly as control populations (Conney and Burns, 1972). The literature on the interaction of drugs is overwhelming as exemplified by that on cimetidine, the new histamine H_2-receptors blocking agent.

Temperature and oxygen tension can clearly influence the rate of drug metabolism. For example, those drugs that are conjugated with glucuronic acid in the liver undergo less conjugation at lower temperatures, and excretion of the drug may be reduced as the body temperature falls below 31°C. Ionizing radiation and ultraviolet radiation are important climatic and geographic factors and may possibly react synergistically (UNSCEAR, 1982). Several drugs and natural products, such as porphyrins and furocoumarins, can sensitize human skin to ultraviolet radiation and, although the mechanisms of action are hypothetical, the practical applications in avoiding sunburn are obvious.

Clearly, occupational exposures to chemicals can exacerbate the effects of drug regimes or life-style habits such as smoking and drinking, as has already been noted. For example, exposure to agents that induce hepatic microsomal enzymes, such as hydrocarbons in gasoline, will enhance the oxidative metabolism of such drugs as antipyrine (Harman *et al.*, 1981). The range of such possible interactions is large and tends to reduce the effectiveness of the drug.

4.3 METHODS FOR STUDYING INTERACTIONS

4.3.1 Approaches

Methods for evaluating mixtures of chemicals are analogous to those for assessing a technical grade product. The components of the mixture must be known and the effects of each component of the mixture determined in a selected battery of toxicological tests which can be conducted in a relatively short period of time. If the sum of the effects of the major components is not equivalent to the effects of the mixture then the difference is attributable to interactions of the individual components and/or to some unidentified component of the mixture.

If the mixtures are highly complex, separation into several fractions is likely to be the initial step. The number of compounds in the mixture to be tested will have to be decreased to a comparatively small number before detailed consideration of interactions can be made. An exception is made when there is evidence that the number of active compounds is small compared with the total number involved.

The individual fractions can then be tested as the original mixture was, to determine in which fraction(s) the activity resides.

If different batches of mixtures yield different results one must consider the possibility that trace contaminants are involved. Difference in starting materials and in manufacturing processes may be the cause of variations in results.

When the mixture is ready for evaluation, *in vivo* and *in vitro* tests may be performed. If *in vitro* methods are used and yield results of significance, the results should then be confirmed *in vivo* to establish relevance to the intact organism.

Examination of interactions can be divided into two approaches. One approach is to test successively binary, tertiary, and mixtures of higher order, focusing on the interactions to be studied. Tests used for this approach must necessarily be simple and rapid because of the comparatively large number of combinations. This approach would be justified and most useful when little is known about the individual components of the mixture.

An alternative approach may be used when sound basic information is available on the kinetics and chemodynamics of the individual components of the mixture. With these data in hand, a hypothesis of the likelihood and character of interactions can be made and tested. Factors to be considered include routes and sequences of exposure, times of observation, dose response, and the quantitative composition of the mixture.

Epidemiological studies and animal experiments have established that diet and nutrition can influence markedly the response to environmental chemicals. For this reason the need to consider the diet and nutritional status of the experimental animal is emphasized to ensure that nutrition does not result in misleading results that are difficult to interpret.

4.3.2 Methods for Detecting Chemical Interactions during the Chemokinetic Phase

Meaningful interpretation of the results obtained by most of the methods described below requires that the measured effects obtained with a mixture be compared with the effects obtained with a single reference or model compound. The reference compound may be chosen with regard to be convenience of measurement; in other instances reference compounds may be a single component of the mixture being studied. Detailed attention is given to *in vitro* methods later in this section, and in Table 4.2 references are provided to important *in vivo* techniques.

4.3.2.1 Absorption

For gastrointestinal tissues, one of the most widely used techniques is that of the everted gut sac. The rate of transfer of the model substrate from the external

Table 4.2 Short-term *in vivo* studies of chemical interactions

Study	End-point	References
1. Inducing agents	$^{14}CO_2$ produced from $^{14}CO_2$-aminopyrine Increased excretion of 6β-hydroxycortisol in urine Isolation of tissues followed by measurement of enzyme activity	Hunter and Chasseaud (1976)
2. Distribution of chemicals	Disposition of radiolabel in whole body sections of rats given radiolabelled chemical	Duprat and Gradiski (1985)
3. Mutagens and promoting agents	See text	Ullberg and Larsson (1981)
4. Hypolipidemic type hepato carcinogens	Isolate liver, determine peroxisome proliferation by electron microscopy or enzyme assay	Lazarow (1978)
5. Early hepatotoxicity[a]	Serum enzymes, bile pigments Tissue assay for enzyme activities Function tests	Schulte-Hermann (1974)
6. Early nephrotoxicity[a]	Enzymes, cells and cell debris in urine Tissue assay for enzyme activities Function tests	Smith and Hook (1982)
7. Early effects on the immune system[a]	Immunity, phagocytosis	Vos (1978)
8. General tissue changes[a]	Whole body monitoring using NMR	Bottomley (1979)
9. Early CNS effects[a]	Behavioural studies	Mitchell and Tilson (1982)

[a] References to histological approach may be found in standard monographs. Use of other tests are described in other sections of this volume.

perfusate (mucosal surface) into the sac (serosal surface) is compared in the absence and presence of the chemicals. Where carrier-mediated processes are suspected of being involved, adequate nutrients must be provided to ensure sufficient energy production to sustain the process (Bridges and Bach, this volume). An analogous procedure has been developed with skin slices which can be used to assess interactions in absorption through this tissue.

4.3.2.2 Tissue Distribution and Extracellular Protein Binding

Some indications of interactions between chemicals leading to changes in the distribution of the chemicals in the tissue or the rates of distribution may be obtained using perfused organ techniques (e.g. in the liver, kidney, and lung). Although the rate of uptake of a model chemical by the tissue may be modified by the presence of other chemicals, the interpretation that this is purely a phenomenon of distribution may require further study and support, since interactions other than the competition for cellular binding sites may occur in the course of metabolism and excretion from the tissue. The nature of the perfusate may further complicate the interpretation since the use of blood, modified blood, or plasma can introduce an additional factor of interaction that is associated with binding to plasma proteins.

Several methods are available for investigating the competitive, reversible interaction between chemicals for the binding to plasma proteins, particularly albumins. The best methods are those that cause a minimum disturbance of the equilibrium between protein-bound chemicals and free, non-protein-bound chemicals in solution. This criterion is met by equilibrium dialysis, gel filtration, ultracentrifugation, and diafiltration (for a review see Bridges and Wilson, 1976). These methods can also be used to investigate interactions in reversible binding to cellular proteins.

The study of interactions in covalent binding to proteins and other tissue macromolecules usually requires a radiolabelled reference compound and a tissue preparation capable of metabolizing the radiolabelled chemical to its reactive intermediates.

4.3.2.3 Metabolic Transformations

The effect of one or more chemicals on the metabolic transformation (biotransformation) of a reference chemical can be investigated in perfused tissue preparations, tissue slices, cell suspensions or cultures, tissue homogenates, subcellular fractions, or with purified enzymes (see below, and Bridges and Bach, this volume). The mechanism of interaction, often a form of inhibition, can usually be inferred from the kinetic data obtained in the simplest metabolizing system.

Similar studies performed in animals previously exposed to a mixture of chemicals for varying time periods will reveal whether changes in the metabolic capability have occurred by enzyme destruction or by the induced synthesis of new enzymes. The nature of induced isoenzymic forms of cytochrome P-450 can often be inferred by their spectral characteristics, the specific metabolic pathways catalysed, the form of model substrates, and the inhibitory characteristics of the enzyme. A significant change in the amount of enzymes in the tissue would be a clear indication that interactions have occurred or would occur.

The site of the metabolism of most exogenous chemicals is the liver, and the liver is, therefore, the tissue most often studied. There are several ways this tissue can be investigated, each having a different degree of relevance to the situation in the whole animal.

The Intact Liver In Situ When a combination of exogenous chemicals is administered into the hepatic portal vein, most metabolites are excreted in a concentrated solution in the bile. They can be analysed by direct application of bile to high-performance liquid chromatography (HPLC), provided the metabolites can be detected (e.g. by their radioactivity, absorption of light, fluorescence). This has the advantage that the liver is virtually undisturbed and functions normally, and that sufficient amounts of metabolites are formed for their chemical characterization by microchemical means, mass spectrometry, or high-resolution nuclear magnetic resonance (NMR).

The Perfused Liver The use of a perfused liver is more artificial than the *in situ* preparation, but this technique has the advantage that the composition of the mixtures of chemicals bathing the sinusoids can be controlled. Furthermore, the metabolites can also be measured in those species in which significant amounts of metabolites are excreted through the sinusoids.

Isolated Hepatocytes This is an even less physiological condition than the use of the intact tissue because the tight junctions between cells are broken and repaired during the isolation of the hepatocytes, and the cells exist in a different environment than the normal tissue architecture. Isolated cells have experimental advantages as only small amounts of material are required.

Dividing Hepatocytes in Culture This method is not recommended for general studies because dividing hepatocytes in culture are usually incompletely or abnormally differentiated.

Cell Subfractions These fractions are of use mainly in preliminary studies. The physiological relevance of the data from such preparations must be considered with caution because exogenous metabolism often occurs in several organelles with specific relationships to each other with respect to architecture and

composition. Subfractions are often useful because they produce sufficient quantities of metabolite for chemical characterization.

Extrahepatic Tissues and Organs Extrahepatic organs are not usually studied *in situ* as is the liver, but are sometimes perused (lung or adrenal gland). Isolated cells are often used, particularly when an organ is composed of several different types of cells (e.g. testis) and when the number of cells is small in relation to the organ (e.g. epithelial cells of the bladder). Cell culture is sometimes used when differentiation can be ensured (e.g. keratinocytes). One of the best ways of ensuring normal differentiation *in vitro* is by using organ culture, but the detection of metabolites is often limited.

4.3.2.4 Excretion

The use of *in vitro* methods for the study of excretion is limited. Perfused liver and kidney preparations have some value and can be used to study interactions between chemicals in the excretion process. The uptake by tissue slices of chemicals that are actively secreted also provides a model for investigating the effects of other compounds on some aspects of the excretion processes (see Bridges and Bach, this volume).

4.3.3 Methods for Detecting Chemical Interactions during the Chemodynamic Phase

4.3.3.1 Mutagenicity and Short-term Tests

Systems that may be used for detecting the effects of chemical interactions at genetic end-points are listed in Table 4.3, including those more commonly used with complex mixtures (see B1, B3, C1, and D5 of Table 4.3). However, these assays must be used with caution if the objective is to study the mechanism of interaction of chemicals. They can be used as indicators of biologically significant events and to signal the modulation of an effect by other chemicals, but these assays shed no light on where, in a series of events, interaction has occurred. Other difficulties are: (a) distribution problems may be minimized in *in vitro* assays but they are not eliminated; (b) movement of molecules across membranes is still required but may not occur with equal facility by components of a mixture; and (c) certain assays are insensitive or not yet adequately validated (see, for example, A2, A4, D1, D3, and D4 of Table 4.3). For sister chromatid exchange, the significance of the biological end-point is unknown.

Cell transformation and initiation/promotion effects also are detectable *in vitro*. Initiation/promotion is a particularly interesting phenomenon in this context since it is a clear example of the interaction of chemicals in a biological system. To test for carcinogens the following cells have been used: Syrian hamster

Table 4.3 Summary of systems used for detecting effects of chemicals at genetic end-points

A. *DNA repair*
   1. Bacteria
   2. Mammalian cells in culture
   3. Hepatocytes
   4. *In vivo/in vitro* hepatocyte assay

B. *Mutations*
   1. Bacteria
   2. Host-mediated assay
   3. Mammalian cells

C. *Chromosome damage*
   1. Mammalian cells
   2. Plant cells

D. *Miscellaneous*
   1. Gene conversion in yeast (Zimmerman, 1977)
   2. Mitotic recombination in yeast
   3. Aneuploidy in *Sordaria*
   4. Aneuploidy in yeast
   5. Sister chromatid exchange (Latt *et al.*, 1977, 1981)

embryo cells (DiPaolo and Casto, 1977; Pienta, 1979), BALB/C-3T3 cells (Kakunaga and Crow, 1980), and C3H/1OT1/2 cells (Reznikoff *et al.*, 1973). A review of these methods can be found in Heidelberger *et al.* (1983).

4.3.3.2 Measurement of Other Interactions

Measurements of chemical interactions resulting in effects other than mutagenicity are normally carried out using intact cells or tissues.

Nucleic Acids and Proteins Whereas several metabolites obtained *in vitro* may be mutagenic and form DNA adducts, only a select few form adducts *in vivo* (for example, several benzo[*a*]pyrene epoxides are mutagenic *in vitro*, but only the (+) anti-BPDE (benzo[*a*]pyrene dihydrodiolepoxide) adduct is observed *in vivo*). Similar considerations apply to proteins. The difference between protein binding *in vitro* and *in vivo* is often striking, particularly with microsomal proteins.

With both proteins and nucleic acids, the direct measurement of the binding observed is determined by hydrolysis and isolation of the chemical adduct by HPLC, followed by identification using microchemical techniques, mass spectrometry, and high-resolution NMR. Sometimes, binding is deduced to have occurred as a result of the loss of activity such as the loss of RNA polymerase II activity.

Cell Membranes Oxidative damage to cell membranes can be determined *in vivo* by the measurement of exhaled ethane or of diene conjugation on sacrifice. In the bile duct of a cannulated animal, hepatic membrane oxidation can be inferred by biliary GSSH levels.

In vitro damage to cell membranes is determined by the appearance of malondialdehyde formation or by a disproportionate loss of polyunsaturated fatty acids on membrane hydrolysis.

4.3.3.3 Measurement of Chemical Interactions in Plants

There are advantages and disadvantages of *in vitro* biochemical studies of plants. There is a restricted range of suitable species for detailed study, principally because many of the secondary products (e.g. phenolics) interfere with the isolation and assay procedures. On the other hand, less concern has to be paid to organ–tissue relationships. In a leaf, for example, well over 90 % of the cells are of the mesophyll type and vascular elements can easily be removed. The presence of rigid cellulose walls does not present problems and protoplasts can readily be prepared. Normal subcellular fractionations which rely on differential or density gradient centrifugation are well developed, and specialized techniques such as filtering silicone-oil centrifugation, which were originally applied to animal mitochondrial studies, have been extensively developed and improved in studies of plant organelles (Hampp, 1980).

By the combined use of protoplasts and filtering silicone-oil centrifugation, the metabolic properties of whole plants, cells, or organelles (mitochondria and plastids) can be determined by procedures which rapidly separate cellular compartments within seconds. Non-aqueous procedures for organelle isolation are also available (Stocking, 1959). These are particularly useful in studies of water-soluble pollutants and their products. By applying suitable diagnostic analysis, the concentrations of the products of interacting pollutants may be determined. In the case of sulphur dioxide and nitrogen dioxide fumigation of plants, high-performance ion chromatography is being used to establish interacting levels of sulphite and nitrite within chloroplasts isolated by aqueous and non-aqueous means (Wellburn, 1985).

Equivalent ranges of pollutants or their products may then be tested *in vitro* with cellular preparations isolated from unpolluted tissue. For whole bio-energetic organelles or detached membrane systems, a series of analytical procedures is available to assay the effects. The choice is determined intuitively or by a process of elimination, assuming adequate sensitivity of the procedure. In the case of wet or dry deposition on plants of acidic substances such as sulphur dioxide and nitrogen dioxide, changes of internal cellular pH levels are likely, and methods appropriate to internal pH measurement are applied. Chemical shifts of the ^{31}P-NMR signals of orthophosphate can be used effectively on organelles (mitochondria or plastids) or protoplasts to measure buffering capacity (Foyer *et*

al., 1982) or, alternatively, the quenching of fluorescent dyes such as 9-aminoacridine can be used as a means of determining pH across membranes, including those engaged in photosynthetic or oxidative electron transport (Robinson and Wellburn, 1983). Deterioration of the efficiency of such membranes by interactive processes leads to reductions of ATP and other phosphorylated intermediates which can be assayed by bioluminescence or by ^{31}P-NMR; at the same time the products of free-radical agents may be monitored by the characteristic evolution of ethane and the reduction in levels of GSH, ascorbate, and other scavengers such as tocopherol (Halliwell, 1982). Other characteristic signs of damage in energetic membranes which lead to the ejection of critical ions such as manganese or iron may be detected by electron-spin resonance (Rowlands *et al.*, 1977).

In plants, an additional, interesting technique has emerged which may have application to microbial and vertebrate studies. It is possible in a variety of plant species to select organisms that are sensitive to or tolerant of a single pollutant (e.g. sulphur dioxide) and to clone them to have sufficient numbers for experimental purposes. These sensitive or tolerant plants may be tested either in the field or in the laboratory in an *in vivo* or an *in vitro* manner to elucidate the mechanisms involved in sensitivity. Recent work has indicated the possibility that individual organisms showing sensitivity or tolerance to interactions (e.g. sulphur dioxide + nitrogen dioxide) may also be selected and then used in a similar manner to help elucidate the mechanisms involved in the interaction (Wellburn *et al.*, 1981).

4.4 CONCLUSIONS

(1) Man is exposed to a very large number of chemicals, each of which has the potential to affect the toxicity of any other. The number of potential interactions is therefore vast. It is impractical to test every possible combination of chemicals for potentially significant interactions. Moreover, even if such an approach was adopted, our present conventional methods for toxicity testing would not be appropriate because of costs, time-scale and relevance. There are comparatively few examples of major interactions between pollutants, but it is not clear whether this indicates the infrequency of such events, or simply failure to detect them.

(2) An alternative approach is to identify, by research, the most common pathways in which major interactions occur. Individual chemicals and biological and physical agents can then be assessed for their potency in modulating these pathways, and thereby their likely interaction potential *in vivo* can be evaluated. By placing emphasis on mechanistic research, ultimately the prediction of such interactions may become sufficiently developed so that the priorities for assessing the potential for interaction can be identified from physicochemical considerations alone.

(3) It is helpful to divide the toxic processes into three phases when considering mechanisms of interaction: pre-absorption, chemokinetics, and

chemodynamics. To date, the greatest progress has been made in understanding interactions in the chemokinetic phase, particularly the interactions involving cytochrome P-450. Certain properties have emerged as increasing the likelihood of interactions in the chemokinetic phase, namely:

(a) long biological half-time;
(b) potent inducing agents of microsomal MFOs;
(c) strong inhibitors of microsomal MFOs;
(d) toxic action resulting from reactive metabolite formation; and
(e) structural similarity to chemical(s) known to cause interactions.

(4) When interactions are identified or predicted, biochemical studies of the nature of the mechanisms involved should be carried out in parallel with monitoring and epidemiological studies. At the same time the hazard may be alleviated by a variety of approaches which include restricting the source of the pollutant. To optimize progress more emphasis should be placed on:

(a) Identification and characterization of the mechanisms underlying strong synergistic interactions. This type of work must continue to be supported, even if the particular chemicals of concern cease to be of practical importance, because of its potential for predicting other interactions and improving the elucidation of new problems.
(b) Examination of likely mechanisms of potent interactions, such as active-transport systems such as Ca^{2+} transport, cell-surface receptors for hormone growth factors, and endo- and exocytosis. It is important that in conducting such mechanistic studies attention is directed to important extrahepatic targets for toxicity, for example neurones and β-cells of the pancreas. Such information may be important for understanding the causes of the increasing incidence of some human diseases such as diabetes.
(c) The establishment, by meticulous observations, of the frequency and magnitude of interactions.

(5) For several economically important species, particularly plants, identifying and understanding the mechanisms of interaction may improve the selection of strains resistant to pollutants, especially species that have hitherto shown little tolerance.

(6) It is important that interactions that have been characterized by using *in vitro* techniques are also confirmed *in vivo* to establish their relevance.

4.5 RECOMMENDATIONS

(1) When developing methods to assess the toxicity of chemicals, increased attention should be paid to studies of kinetics and mechanisms of action. This will provide a sound basis for the interpretation of possible interactions.

(2) Studies of chemical interactions at target cells other than those of the liver should be intensified, with particular attention given to those involved in fertility and human disease.

(3) Studies of chemical interactions should be broadened from the MFO system to systems such as those affecting the cellular concentration of calcium, the growth factor, cell-surface receptors, active-transport mechanisms, endo- and exocytosis, and the transport of proteins.

(4) Even when a chemical interaction has been identified and its consequences avoided by removal of some components of the mixture, further research should be undertaken to elucidate the underlying mechanism of interaction because of the likely relevance to other interaction processes. The results could lead to important practical applications.

(5) In any *in vivo* toxicological investigation, care should be taken to identify to the greatest possible extent the potential interacting components in, for example, diets, chemicals under test (impurities), and testing media (e.g. water).

(6) Pollution sensitive and tolerant organisms should be used as indicator species to identify the genetic factors associated with mechanisms of chemical interaction. Once this is established, application in cloning, gene splicing and conventional plant breeding is possible.

(7) Because some plants have a high genetic potential for tolerating particular pollutants, these adaptations should be used in polluted areas even before the mechanisms of resistance are understood.

4.6 REFERENCES

Ariens, E. J. (1972). Adverse drug interactions. *Proc. Eur. Soc. Study Drug Toxicol.*, **13,** 37–63.

Babson, J. R., and Reed, D. J. (1978). Inactivation of glutathione reductase by 2-chloroethyl nitrosourea-derived isocyanates. *Biochem. Biophys. Res. Commun.*, **83,** 754–762.

Bottomley, P. A. (1979). *In vivo* tumour discrimination in a rat by proton nuclear magnetic resonance. *Cancer Res.*, **39,** 468–470.

Bridges, J. W., and Wilson, A. G. E. (1976). Drug–serum protein interactions and their biological significance. In Bridges, J. W., and Chasseaud, L. F. (Eds.) *Progress in Drug Metabolism*, Vol. 1, pp. 193–247. John Wiley & Sons, Chichester.

Brock, T. D. (1970). *Biology of Microorganisms*. Prentice-Hall, Englewood Cliffs, New Jersey.

Conney, A. H. (1982). Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G. H. A. Clowes memorial lecture. *Cancer Res.*, **42,** 4875–4917.

Conney, A. H., and Burns, J. J. (1972). Metabolic interactions among environmental chemicals and drugs. *Science*, **178,** 576–586.

Damber, L., and Larsson, L. G. (1982). Combined effects of mining and smoking in the causation of lung carcinoma. *Acta Radiol. Oncol.*, **21,** 305–313.

DiPaolo, J. A., and Casto, B. C. (1977). *Chemical Carcinogenesis*, pp. 17–47. CRC Press, Cleveland, Ohio.

Doll, R., and Peto, R. (1981). The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J. Natl. Cancer Inst.*, **66,** 1191–1308.

DuBois, K. P. (1961). Potentiation of the toxicity of organophosphorus compounds. *Adv. Pestic. Control Res.*, **4,** 117–151.

Duprat, P., and Gradiski, D. (1985). Autoradiographic distribution of hexachlorobutadiene. Study in the rat after single epicutaneous application. *Toxicologist*, **5(1),** 54.

Foyer, C., Walker, D., Spencer, C., and Mann, B. (1982). Observations on the phosphate status and intracellular pH of intact cells, protoplasts, and chloroplasts from photosynthetic tissue using phosphorous-31 nuclear magnetic resonance. *Biochem. J.*, **202,** 429–434.

Galloway, D. A., and McDougall, J. K. (1983). The oncogenic potential of herpes simplex viruses: evidence for a 'hit-and-run' mechanism. *Nature*, **302,** 21–24.

Greenwald, P. C. (1984). Personal communication, National Cancer Institute, Bethesda, Maryland.

Grice, H. C., Wiberg, G. S., and Heggtveit, H. A. (1981). Studies in food additive cardiopathies. In Balazs, T. (Ed.) *Cardiac Toxicology*, Vol. II, pp. 189–201. CRC Press, Boca Raton, Florida.

Halliwell, B. (1982). Super oxide and super-oxide dependent formation of hydroxyl radicals are important in oxygen toxicity. *Trends Biochem. Sci.*, **7,** 270–272.

Hamagami, C. M., Tsuchiya, K., and Kingsted, S. (1978). *Cadmium Studies in Japan: Review*. Elsevier, Tokyo: 376 pages.

Hampp, R. (1980). Rapid separation of the plastid, mitochondrial and cytoplasmic fractions from the intact leaf protoplasts of *Avena*. *Planta*, **150,** 291–298.

Harman, A. W., Frewin, D. B., and Priestly, B. G. (1981). Induction of microsomal drug metabolism in man and in the rat by exposure to petroleum. *Br. J. Ind. Med.*, **38,** 91–97.

Heidelberger, C., Freeman, A. E., Pienta, R. J., Sivak, A., Bertram, J. S., Casto, B. C., Dunkel, V. C., Francis, M. W., Kakunaga, T., Little, J. B., and Schechtman, I. M. (1983). Cell transformation by chemical agents—a review and analysis of the literature. *Mutat. Res.*, **114,** 283–385.

Hoyumpa, A. M., and Schenker, S. (1982). Major liver interactions: effect of liver disease, alcohol, and malnutrition. *Annu. Rev. Med.*, **33,** 113–149.

Hunter, J. O., and Chasseaud, L. F. (1976). Clinical aspects of microsomal enzyme induction. In Bridges, J. W., and Chasseaud, L. F. (Eds.) *Progress in Drug Metabolism*, Vol. 1, pp. 129–192. John Wiley & Sons, Chichester.

Ivankovic, S., Preussmann, R., Schmahl, D., and Zeller, J. W. (1975). Prevention by ascorbic acid of *in vivo* formation of *N*-nitroso compounds. In Bogovski, P., and Walker, E. A. (Eds.) *N-Nitroso Compounds in the Environment*. IARC Scientific Publications No. 9, pp. 101–102. International Agency for Research on Cancer, Lyon.

Ivanovic, V., and Weinstein, B. (1981). Glucocorticoids and benzo[*a*]pyrene have opposing effects on EGF receptor binding. *Nature*, **293,** 404–406.

Kakunaga, T., and Crow, J. D. (1980) Cell variants showing differential susceptibility to UV light induced transformation. *Science*, **209,** 505–507.

Koziol, M. J., and Whatley, F. R. (1984). *Gaseous Air Pollutants and Plant Metabolism*. Butterworths, London.

Latt, S. A., Allen, J. W., Rogers, W. E., and Juergens, L. A. (1977). *In vitro* and *in vivo* analysis of sister chromatid exchange formation. In Kilbey, B. J., Legator, M., Nichols, W., and Ramel, C. (Eds.) *Handbook of Mutagenicity Test Procedures*, pp. 275–291. Elsevier, Amsterdam.

Latt, S. A., Allen, J., Bloom, S. W., Carrano, A., Falke, E., Kram, D., Schneider, E., Schreck, R., Tice, R., Whitfield, B., and Wolff, S. (1981). Sister-chromatid exchanges: a report of the Gene-Tox Programme. *Mutat. Res.*, **87,** 17–62.

Lazarow, P. B. (1978). Rat liver peroxisomes catalyze the β-oxidation of fatty acids. *J. Biol. Chem.*, **253,** 1522–1528.

McDonald, J. C. (1980). Asbestos-related disease: an epidemiological review. In Wagner, J. C. (Ed.) *Biological Effects of Mineral Fibres.* IARC Scientific Publications No. 30, Vol. 2, pp. 587–601. International Agency for Research on Cancer, Lyon.

Meredith, M. J., and Reed, D. J. (1982). Status of the mitochondrial pool of glutathione in the isolated hepatocyte. *J. Biol. Chem.*, **257,** 3747–3753.

Metcalf, R. L. (1967). Mode of action of insecticide synergists. *Annu. Rev. Entomol.*, **12,** 229–256.

Mirvish, G. S. (1981). Inhibition of the formation of carcinogenic *N*-nitroso compounds by ascorbic acid and other compounds. In Burchenal, J. H., and Oettgen, H. F. (Eds.) *Cancer: Achievements, Challenges and Prospects for the* 1980's, Vol. 1, pp. 557–587. Grune and Stratton, New York.

Mitchell, C. L., and Tilson, H. A. (1982). Behavioural toxicology in risk assessment: problems and needs. *CRC Crit. Rev. Toxicol.*, **10,** 265–274.

NAS/NRC (1980). *Drinking Water and Health*, Vol. 3, pp. 265–403. National Academy Press, Washington, DC.

NAS/NRC (1982). *Diet, Nutrition and Cancer*, pp. 10/1–10/40. National Academy Press, Washington, DC.

Ohshima, H., and Bartsch, H. (1981). Quantitative estimation of endogenous nitrosation in humans by monitoring *N*-nitrosoproline excreted in the urine. *Cancer Res.*, **41,** 3658–3662.

Ormrod, D. P. (1982). Air pollutant interactions in mixtures. In Unsworth, M. H., and Ormrod, D. P. (Eds.) *Effects of Gaseous Air Pollutants in Agriculture and Horticulture*, pp. 307–331. Butterworth Scientific, London.

Pelletier, O. (1975). Vitamin C and cigarette smokers. *Ann. N.Y. Acad. Sci.*, **258,** 156–168.

Pienta, R. J. (1979). A hamster embryo cell model system for identifying carcinogens. In Griffin, A. C., and Shaw, C. R. (Eds.) *Carcinogens: Identification and Mechanisms of Action*, pp. 121–141. Raven Press, New York.

Reznikoff, C. A., Bertram, J. S., Brankow, D. W., and Heidelberger, C. (1973). Quantitative and qualitative studies of chemical transformation of cloned C-3-H mouse embryo cells sensitive to post confluence inhibition of cell division. *Cancer Res.* **33,** 3239–3249.

Robinson, D. C., and Wellburn, A. R. (1983). Light-induced changes in the quenching of 9-amino-acridine fluorescence of photosynthetic membranes due to atmospheric pollutants and their products. *Environ. Pollut.*, **32,** 109–120.

Rowlands, J. R., Allen-Rowlands, C. J., and Gause, E. M. (1977). Effects of environmental agents on membrane dynamics. In Lee, S. D. (Ed.) *Biochemical Effects of Environmental Pollutants*, pp. 203–246. Ann Arbor Science, Ann Arbor, Michigan.

Schulte-Hermann, R. (1974). Induction of liver growth by xenobiotic compounds and other stimuli. *CRC Crit. Rev. Toxicol.*, **3,** 97–158.

Schwab, M., Alitalo, K., Varmus, H. E., and Bishop, J. M. (1983). A cellular oncogene (*c-Ki-ras*) is amplified, overexpressed, and located within karyotypic abnormalities in mouse adrenocortical tumour cells. *Nature*, **303,** 497–501.

Smith, J. H., and Hook, J. B. (1982). Experimental nephrotoxicity *in vivo*. In Bach, P. H., Bonner, F. W., Bridges, J. W., and Lock, E. A. (Eds.) *Nephrotoxicity. Assessment and Pathogenesis*, pp. 117–127. John Wiley & Sons, Chichester.

Sporn, M. B., and Newton, D. L. (1981). Recent advances in the use of retinoids for cancer prevention. In Burchenal, J. H., and Oettgen, H. R. (Eds.) *Cancer: Achievements, Challenges and Prospects for the* 1980's, Vol. 1, pp. 541–548. Grune and Stratton, New York.

Stocking, C. R. (1959). Chloroplast isolation in non-aqueous media. *Plant Physiol.*, **34,** 56–61.

Tannenbaum, S. R., Jett, D., Young, V. R., Land, P. D., and Bruce, W. R. (1978). Nitrite and

nitrate are formed by endogenous synthesis in the human intestine. *Science*, **200,** 1487–1489.

Ullberg, S., and Larsson, B. (1981). Whole body autoradiography. In Bridges, J. W., and Chasseaud, L. F. (Eds.) *Progress in Drug Metabolism*, Vol. 6, pp. 249–289. John Wiley & Sons, Chichester.

UNSCEAR (1982). *Ionizing Radiation: Sources and Biological Effects*. United Nations Scientific Committee on the Effects of Atomic Radiation, 1982 Report to the General Assembly, 37th Session, Supplement No. 45 (A/37/45). United Nations, New York: 773 pages.

Vos, J. G. (1978). Immune suppression as related to toxicology. *CRC Crit. Rev. Toxicol.*, **5,** 67–101.

Walker, C. H. (1980). Species variations in some hepatic microsomal enzymes that metabolize xenobiotics. In Bridges, J. W., and Chasseaud, L. F. (Eds.) *Progress in Drug Metabolism*, Vol. 5, pp. 113–164. John Wiley & Sons, Chichester.

Wellburn, A. R. (1985). Ion chromatographic determination of levels of anions in plastics from fumigated and non-fumigated barley seedlings. *New Phytol.*, **100,** 329–339.

Wellburn, A. R., Higginson, C., Robinson, D. C., and Walmsley, C. (1981). Biochemical explanations of more than additive inhibitory effects of low atmospheric levels of sulphur dioxide plus nitrogen dioxide upon plants. *New Phytol.*, **88,** 223–237.

Zimmerman, F. K. (1977). Procedures used in the induction of mitotic recombination and mutation in the yeast *Saccharomyces cerevisiae*. In Kilbey, B. J., Legator, M., Nichols, W., and Ramel, C. (Eds.) *Handbook of Mutagenicity Test Procedures*, pp. 119–134. Elsevier, Amsterdam.