Methods for Assessing the Effects of Mixtures of Chemicals
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Induction, Inhibition and Receptor Interactions

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1 INTRODUCTION

In real life, man is exposed simultaneously to a plethora of naturally occurring chemical compounds present in the general environment and in food. Modern life has added to these in the development and use of food additives, pesticides, and drugs, and exposure to a range of chemical agents in different working environments. The expectation is that these compounds will interact in a variety of ways by a variety of mechanisms with the resultant summation, synergism, or antagonism of their respective individual effects and toxicities. Even if the identity of all the components in a given exposure were known, the study of the possible combinations of the individual components presents an unrealistic task, particularly when it is appreciated that many of the components may not be treated as independent variables for such processes as metabolism, receptor interaction, etc. The approach to the biochemical aspects of toxicology has therefore been in accordance with the established scientific method of studying each factor separately so that the variables can be controlled. This has led to a sound understanding of the processes involved in the absorption, distribution, metabolism, and excretion for a large number of chemical compounds and a good understanding in many instances of the mechanisms by which these processes are accomplished.

With regard to the specific aspects of enzyme induction and inhibition, and receptor interactions, studies have largely been confined to mixtures of two components, i.e. the effect of one component either on the effect produced by the other, or on the rate of metabolism of the other. In enzyme induction studies, this has usually been the effect of pretreatment by one agent on the subsequent metabolism of other agents. The interpretation of such studies has relied heavily on an understanding of the mechanism of the processes under investigation. Such knowledge will be equally vital in predicting and interpreting effects observed from the study of more complex mixtures.

2 GENERAL CONSIDERATIONS

The overall fate of an exogenous, non-nutritive chemical compound (xenobiotic) can be represented schematically as presented in Figure 1. Interactions between compounds can occur at any of the three phases—the exposure phase, the kinetic phase, and the response phase. In the kinetic phase, such interactions are due to changes produced in the specific processes of absorption, plasma protein binding, metabolism, or excretion. With regard to absorption, such changes may be because of competition for absorption (rarely), alterations in the membrane, or interaction between individual compounds with a resulting change in their physical characteristics, e.g. lipophilicity. Competition for binding to specific carrier proteins or enzymes results in interactions in plasma protein binding and decreased rates of excretion and metabolism. Metabolism may also be altered by the induced synthesis of the normally occurring forms of enzymes or new forms of the enzymes with different metabolic characteristics.

In the response phase, interactions at receptor sites in the target tissues may result in enhanced, diminished, or different effects as a result of synergism or antagonism.

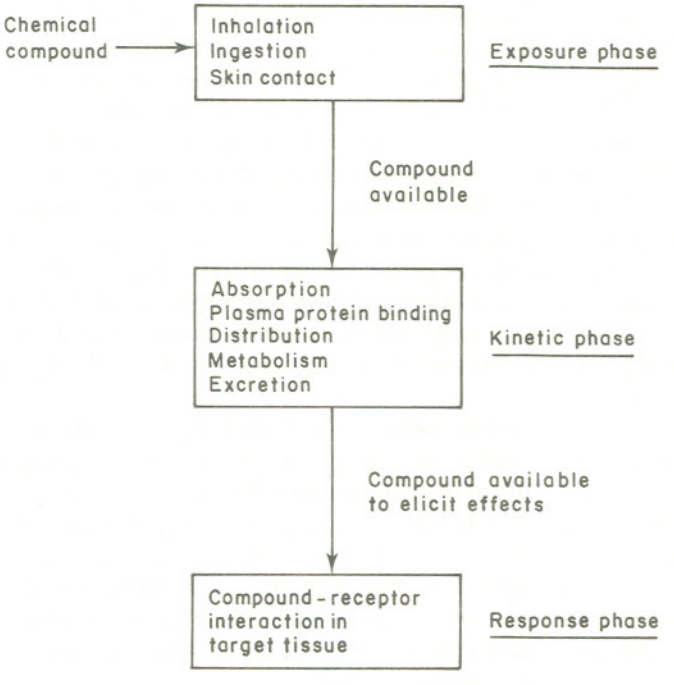

Figure 1 Overall fate of a xenobiotic

3 ABSORPTION AND DISTRIBUTION

For the vast majority of xenobiotics, the mechanism of the processes of absorption and distribution within tissues is that of passive diffusion of lipophilic molecules across a lipophilic membrane as described by Fick's Law:

$$\text{Rate of transfer} = PA \times \text{concentration difference across membrane}$$

where A = surface area of the membrane, P = a 'biological partition coefficient' or penetration rate constant.

Since the surface area is very large, it is extremely unlikely that this parameter becomes rate limiting in the passage of a mixture of compounds across the membrane. The penetration rate constant depends largely upon the lipophilicity of the compound and the nature of the membrane. Thus the presence of one compound will not alter the rate of transfer across the membrane of another compound unless one component causes an alteration in the membrane structure or, when present together, the two components combine to form a more lipophilic species, e.g. ion pair formation.

Hexamethonium, a quaternary ammonium drug containing two positively charged nitrogen atoms, is variably absorbed after oral dosing in man. This absorption, which would not be expected on the basis of passive diffusion through a lipid membrane, is attributed to formation of a more lipid-soluble ion pair with endogenous anions present in the gastrointestinal tract.

An example of altered membrane permeability, possibly as a result of physical damage to the membrane, may be afforded by the observation that polycyclic hydrocarbons may enter cells more readily, and are more extensively taken up by liver microsomes, when associated with asbestos fibres and other particulate materials. This physical interaction of these components greatly increases the toxicity and carcinogenicity of these agents (Lakowicz and Bevan, 1979).

3.1 Plasma Protein Binding

When present in blood, many lipophilic compounds, particularly weakly anionic compounds, are bound non-covalently to plasma proteins, principally albumin. For many compounds, the nature of the interaction with albumin is complex and can be represented as a receptor protein with a number of binding sites. The usual pattern of the interaction is that of reversible equilibrium with one binding site, or a limited number of binding sites, of high affinity on the protein molecule and a greater number of low-affinity sites:

$$\underset{\text{xenobiotic}}{\text{Albumin} + n_1\text{X}} \rightleftharpoons \underset{\text{protein-bound xenobiotic}}{\text{Albumin} - n_1\text{X}}$$

for n_1 binding sites of high affinity

$$\text{Albumin} - n_1\text{X} + n_2\text{X} \rightleftharpoons \begin{array}{l}\text{Albumin} - n_1\text{X} \\ \quad\vert \\ \quad n_2\text{X}\end{array}$$

for n_2 binding sites of low affinity

An example of this phenomenon is afforded by the albumin binding of the fluorescent compound dansylglycine (Figure 2) (Chignell, 1969). Analysis of this Scatchard plot of the equilibrium dialysis data indicates that there is one high-affinity binding site of association constant 4.6×10^5 and a greater number of binding sites of lower affinity with association constants of less than 1×10^5. On binding to albumin, dansylglycine undergoes a change in its fluorescence characteristics; Figure 2 shows that this change is associated solely with binding to the high-affinity site which has been characterized as a hydrophobic region in the albumin molecule.

When more than one compound is present in blood with the ability to bind to the same site on albumin, competition for binding, or displacement of that molecule already bound, may occur. This phenomenon is illustrated in Figure 3 which shows that phenylbutazone competitively displaces dansylglycine from its high-affinity, hydrophobic binding site on albumin. In other experiments, the association constant of phenylbutazone for the hydrophobic site on albumin has been estimated as 1×10^5 and therefore competition for binding would be anticipated. Several such interactions have been recognized as being of clinical significance in multiple drug therapy (Table 1).

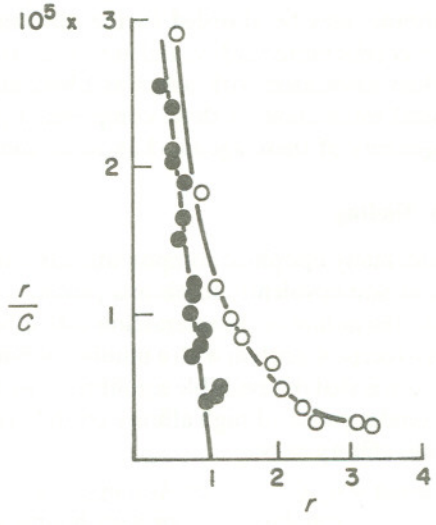

Figure 2 Scatchard plot of the binding of dansylglycine to human serum albumin. Results were obtained from equilibrium dialysis experiments (○) or from fluorescence titration measurements (●). r = number of moles of dansylglycine bound per mole of human serum albumin. C = molar concentration of free, non-protein-bound, dansylglycine. Adapted from Chignell (1969)

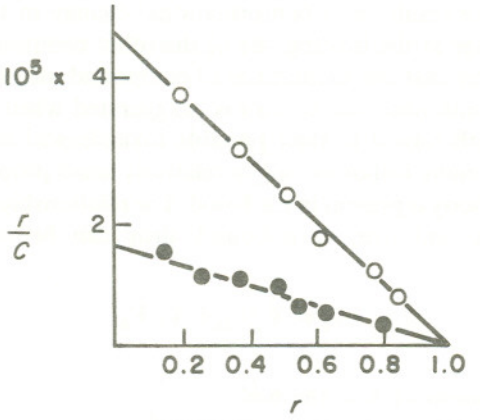

Figure 3 Scatchard plot of the binding of dansylglycine to human plasma albumin in the presence of phenylbutazone. C = molar concentration of free, non-protein-bound dansylglycine. r = number of moles of dansylglycine bound per mole of albumin. Binding was measured by monitoring the increase in dansylglycine fluorescence. Human albumin alone (1×10^{-5} M) (○); human albumin (1×10^{-5} M) + phenylbutazone (2.5×10^{-5} M) (●). All solutions contained 0.1 M sodium phosphate buffer (pH 7.4). Adapted from Chignell (1969)

Table 1 Interactions due to drug displacement from albumin binding

Strongly bound drug	Drug displaced	Resultant effect
Phenylbutazone, oxyphenbutazone	Coumarin anticoagulants	Enhanced inhibition of prothrombin synthesis resulting in haemorrhage
Coumarin anticoagulants	Sulphonamides	Enhanced bacteriostatic action
Sulphaphenazole	Tolbutamide	Enhanced effects of tolbutamide on the pancreas resulting in increased hypoglycaemia
Sulphonamides, penicillins, salicylates	Bilirubin	Jaundice and kernicterus in children

The ability of individual compounds to bind to albumin should not be interpreted as a clear indication that there will be competition for binding with the protein when they are both present. Interactions will only occur if they compete for the same binding site, as represented in Figure 3, or if there is some form of non-competitive interaction, such that the binding of one compound at

its appropriate site results in a conformational change in the protein with a concomitant change at the binding site of the other compound.

There is a danger that the significance of compound interactions for binding with plasma albumin observed *in vitro* is exaggerated when their implications are considered with regard to their possible toxicological effects *in vivo*. This arises because, in many instances, only a relatively small proportion of the total compound in the body is present in the blood. The relationship between albumin-bound compound and free, or unbound, drug can be represented by the expression

$$X_t = X_u \cdot V_D + X_b \cdot V_B$$

where

X_t = total amount of X in the body;
X_u = concentration of unbound compound;
X_b = concentration of compound in blood, bound to albumin;
V_D = volume of distribution of unbound compound; and
V_B = volume of distribution of bound compound (= volume of blood).

For many lipophilic xenobiotics, V_D is equal to total body water, or a greater volume if it is sequestered, for example in adipose tissue, i.e. $V_D > 10 V_B$. Using the minimum value of $V_D = 10 V_B$ and assuming in the extreme that interaction results in complete displacement from albumin, abolition of 50% binding to albumin results in an increase at equilibrium of the unbound concentration X_u by a factor of 1.1; abolition of 90% binding to albumin gives an increase in X_u by a factor of 1.9. Only when the percentage compound bound to albumin in blood is 99% of the total in blood would complete displacement cause a very significant rise in X_u by a factor of 10.9. Thus it is apparent that significant changes in blood concentration will only occur when the compound displaced is normally very highly protein bound, i.e. in excess of 90% of the total blood content of the compound.

The other circumstance in which displacement of an albumin-bound compound may produce a significant rise in the concentration of unbound drug in the blood is for those compounds which have a volume of distribution substantially less than total body water, i.e. when the blood contains a significant amount of the total compound in the body.

The initial effect of one compound causing displacement of another from its albumin binding site is to cause an increase in the blood concentration of the unbound form of the displaced compound. The displaced compound will distribute rapidly into tissues in accordance with the volume of distribution of the unbound drug. This may result in an increased concentration of the compound at its site of action, sufficient to elicit a significant response as exemplified by the enhanced drug effects given in Table 1.

The general impression given in the foregoing discussion is that the albumin-bound form of a compound is inactive and is unable, in that form, to express a

pharmacological or toxicological effect. This is generally considered to be valid but caution must be exercised in concluding that changes in albumin binding are responsible for the observed effect, as indicated earlier. A good example of this effect is seen in the pretreatment of mice with the insecticides aldrin, dieldrin, DDT, or chlordane which decreased the toxicity of the cholinesterase inhibitor paraoxon (Tables 2 and 3), the decrease being paralleled by a decrease in the concentration of free, non-albumin-bound compound in plasma (Triolo *et al.*, 1970). The 40-fold decrease in plasma concentration of unbound paraoxon between control mice and mice six days after aldrin pretreatment (Table 2), corresponding to an increase of plasma protein binding from 81% to 99.5%, is sufficient to account for the observed toxicity. A change in concentration of free paraoxon of this magnitude is in agreement with the expression given earlier, relating unbound and bound concentrations and total compound in the body, and indicates that the volume of distribution of paraoxon in mice is approximately ten times the plasma volume.

Table 2 Time effect of the protective action of a single dose of aldrin in paraoxon toxicity and plasma paraoxon binding in mice

Time of aldrin dosage before paraoxon (days)	Mortality (%)	Plasma free (unbound) paraoxon (%)
Control: no aldrin	75	19±4
1	55	13±4
2	26	0.8±0.2
4	22	0.6±0.1
6	15	0.5±0.1
12	75	18±1

Data from Triolo *et al.* (1970).

Table 3 Effect of dosage of aldrin on paraoxon toxicity and plasma paraoxon binding in mice

Dose of aldrin (mg/kg)	Mortality (%)	Plasma free (unbound) paraoxon (%)
None	90	15±4
1	75	13±2
2	60	12±4
8	10	1.4±1.2
12	5	1.1±0.3

Data from Triolo *et al.* (1970).

4 INHIBITION OF METABOLISM

Most environmental compounds that are readily absorbed, because of their high lipophilicity, are quickly metabolized into more polar compounds, which enhances their elimination by excretion via the kidneys and bile. The metabolic detoxication of compounds occurs primarily in the liver and is catalysed by a variety of enzymes, especially those found in the endoplasmic reticulum, such as the cytochrome P-450-dependent mixed function oxidases, and subsequently by the glucuronyl and sulphotransferases (Parke, 1982). Most compounds are metabolized by a variety of different enzymic reactions, and the simultaneous ingestion of two or more compounds may lead to their competition for the various enzymic pathways, leading to decreased rates of metabolic transformation. This leads to an enhancement of toxicity in those cases where the parent compound is the toxic entity, but the converse is seen where a metabolite is responsible for the toxicity. Where the concentrations of the various compounds are very low, these competitive enzyme effects will be negligible, but a higher dosage of one or more compounds may lead to marked competition for a particular enzyme, with consequent marked change in chemical toxicity. For example, the simultaneous ingestion of alcohol and various drugs, such as barbiturates, greatly increases the pharmacological activity and toxicity of the latter because of competition for the detoxicating enzymes. In contrast, ethanol ingested immediately following methanol decreases the toxicity of the latter because of competition for the enzyme alcohol dehydrogenase which metabolizes methanol to the highly toxic formaldehyde.

In addition to the complexity that metabolism of a compound may be detoxicating or activating, a further level of complexity is added by the recognition that many of the enzymes responsible for the metabolism of xenobiotics exist in multiple forms or as isoenzymes. This has been well characterized for the cytochrome P-450 class of mixed function oxidases and the conjugating enzymes, the glucuronyl transferases, the sulphotransferases and the glutathione transferases. Although in several instances the isoenzymes are located at the same site within the cell, this is not true in all cases, and with epoxide hydratase and glutathione transferase, forms occur in both the endoplasmic reticulum and the cytosol of the cell. The existence of different substrate specificities for isoenzymes at different cellular locations might reasonably be expected, but this phenomenon is also a characteristic of the isoenzymes found at the same cellular location and is a particular feature of the isoenzymes of the cytochrome P-450 mixed function oxidases. In most instances, the substrate specificities are not absolute, and while one isoenzyme may show a high activity towards a given substrate, the other isoenzymes exhibit lower and varied activities for the same substrate. Thus although competition for metabolism by a mixture of substrates may be anticipated on the basis of common features of their metabolism (e.g. mixed function oxidase-mediated hydroxylation), significant

competition may not be observed in practice because different isoenzymes are concerned with the metabolism of the major individual components of the mixture. Nevertheless, when sufficient amounts of compound are present, such that at least some of the isoenzymes are operating at or near maximum velocity, competition is usually observed probably because of the overlap in the specificities of the isoenzymes.

Since the cytochrome P-450-dependent mixed function oxidases play such a central role in the metabolism of lipophilic xenobiotics, leading in most cases to detoxication but also in a significant number of instances to highly toxic entities, principally this group of enzymes will be considered in the discussion on the mechanism of inhibition of metabolism.

The cytochrome P-450 molecule has two major sites at which xenobiotics can interact with the enzyme: the lipophilic substrate-binding site of the apoenzyme and the oxygen-binding site at the iron atom of the haem prosthetic group. The binding of a substrate to the lipophilic binding site is characterized by a spectral change in the enzyme–substrate complex, termed a type I spectral change. In a mixture of compounds where individual components produce the type I change, the components compete for binding and this is expressed in the enzyme reaction as competitive inhibition of the metabolism of a given substrate. Competition for the binding of oxygen can also occur at the haem-binding site, not only from the classical inhibitors, carbon monoxide and cyanide, but also from a wide range of nitrogen-containing, lipophilic organic compounds. The latter group is characterized by the production of a further spectral change, termed type II, which is attributed to the nitrogen atom acting as a ligand for the iron atom of the haem. Compounds producing a type II effect interact in the metabolism of a given substrate in a reversible, non-competitive manner. In such cases, the assignment of the exact mechanism is often complex since the lipophilic, nitrogen-containing compounds are also able to act as substrates by binding at the substrate binding site. This type of interaction has frequently been termed a mixed inhibition.

Where metabolism leads to the detoxication of a compound, its toxicity may be increased as a result of either competitive or reversible, non-competitive inhibition because of the increased biological half-life of the compound. As a result of inhibition of metabolism, the initial concentration achieved by the compound in the body will be similar to the uninhibited situation but, with the passage of time, higher concentrations of the compound will pertain due to the increased half-life, thus providing a greater potential for the expression of a toxic effect. Where metabolism of the compound results primarily in activation, decreased toxicity would be expected from competitive metabolism.

The metabolism of benzene to phenol, and styrene to phenylglyoxylic, mandelic, and hippuric acids, has been shown to be inhibited by the co-administration of toluene, which competitively inhibits mixed function oxidation and thereby modifies the chemical toxicity (Ikeda *et al.*, 1972). The oxidative metabolism of antipyrine and other drugs may be competitively inhibited by the

simultaneous administration of oral contraceptive steroids (Abernethy and Greenblatt, 1981), alcohol (Reinke *et al.*, 1980), or drugs such as cimetidine (Jackson, 1982), thereby prolonging and increasing their pharmacological activity.

Because of the different specificities of two of the major isoenzymic forms of the haemoprotein mixed function oxidase, the so-called cytochromes P-450 and P-448, competitive inhibition of these two enzymes can be highly specific. The alkaloid ellipticine and its metabolite, 9-hydroxyellipticine, are specific inhibitors (and subsequently inducers) of cytochrome P-448, whereas metyrapone similarly inhibits cytochrome P-450 (Delaforge *et al.*, 1980; Lesca *et al.*, 1978; Phillipson *et al.*, 1982). Consequently, 9-hydroxyellipticine inhibits the Ames test mutagenicity of many carcinogens that are activated by cytochrome P-448 including aflatoxin B_1, benzo[*a*]pyrene, 3-methylcholanthrene, 7,12-dimethylbenz[*a*]-anthracene, 2-acetamidofluorene, *N*,*N*-dimethyl-4-aminoazobenzene, and ethidinium bromide (Table 4) (Lesca *et al.*, 1978). As the carcinogenicity of these potent carcinogens is related to their mutagenicity, it is not surprising that this alkaloid has been used in the prevention and treatment of cancer. Less effective inhibitors of cytochrome P-448 are 7,8-benzoflavone and 3-methylcholanthrene; the carcinogenicity of 2-acetamidofluorene in the rat, which has been attributed to activation by *N*-hydroxylation (cytochrome P-448), is inhibited, as is its *N*-hydroxylation, by simultaneous administration of 3-methylcholanthrene (Razzouk *et al.*, 1980). A less specific inhibitor is the anticancer drug, cimetidine, which shows general inhibition of oxidative drug metabolism, though it does have greater affinity for cytochrome P-448, and effectively protects against paracetamol toxicity (Jackson, 1982) which is specifically activated by cytochrome P-448 (Ioannides *et al.*, 1983).

Competitive inhibition of other enzymes concerned with the metabolism of xenobiotics, leading to the expression of toxicity, is well established. Tri-*o*-tolyl phosphate, an organophosphate cholinesterase inhibitor, protects against the toxic effects of parathion in mice yet slightly enhances the toxicity of paraoxon (Lynch and Coon, 1972) and increases the toxicity of malathion 100-fold (McKay *et al.*, 1971). These effects have been attributed to (a) impaired absorption of parathion, (b) decreased tissue binding of paraoxon, and (c) competitive inhibition of ali-esterase (carboxylesterase) to a much greater extent than that of cholinesterase. In the epidemic of malathion poisoning in 1976, the unexpected toxicity of this pesticide was shown to be related to its content of isomalathion and other impurities, such as the trimethylphosphorothioates, all of which act as competitive inhibitors of carboxylesterase, the enzyme that effects the detoxication of malathion (Verschoyle *et al.*, 1982). The herbicides atrazine, simazine, monuron, and 2,4-dichlorophenoxyacetic acid similarly enhance the toxicity of insecticides several-fold, although the mechanism is unknown (Lichtenstein *et al.*, 1973) (see Table 5).

Non-competitive inhibition of the cytochrome P-450 haemoproteins has also

Table 4 Protective effects of 9-hydroxyellipticine, a specific inhibitor of cytochrome P-448, on the mutagenicity of known carcinogens in the Ames test

Carcinogen	Ames bacterial strain	9-Hydroxyellipticine concentration (μg/plate)			
		0	1	3	5
		Revertants per plate			
Aflatoxin B_1 (80 ng/plate)	TA 98	1000	150	100	150
Ethidinium bromide (1 μg/plate)	TA 1538	1200	400	500	< 50
Benzo[*a*]pyrene (5 μg/plate)	TA 100	750	600	350	50
2-Acetylaminofluorene (5 μg/plate)	TA 1538	1400	650	450	400
3-Methylcholanthrene (10 μg/plate)	TA 100	2800	1800	1200	800
7,12-Dimethylbenz[*a*]anthracene (20 μg/plate)	TA 100	1600	950	650	550
N,*N*-Dimethyl-4-amino-azobenzene (400 μg/plate)	TA 98	–	250	210	80
Cigarette condensate (4 mg/plate)	TA 98	1100	600	400	300

Data from Lesca *et al.* (1978).

Table 5 Synergism of insecticides by herbicides

Insecticide	Concentration of insecticide (μg/jar)	Mortality of *Drosophila melanogaster* (%)		
		No herbicide	Atrazine (40 μg/jar)	Ratio of toxicities
Parathion	0.35	9 ± 8	77 ± 10	8.6
Paraoxon	0.35	13 ± 3	52 ± 3	4.0
Dieldrin	0.14	5 ± 4	36 ± 4	7.2
DDT	2.0	17 ± 15	57 ± 2	3.4
Carbofuran	0.7	43 ± 18	93 ± 5	2.2
Phorate	0.6	22 ± 4	52 ± 7	2.4

Data from Lichtenstein *et al.* (1973).

been shown to occur by a modification of the mechanisms described earlier. Although the parent compound does not bind to the haem iron, an intermediate product, formed during the mixed function oxidation process following binding of the compound to the substrate binding site, provides a suitable ligand for the iron. The formation of such metabolic-intermediate complexes results in non-competitive inhibition of further substrate metabolism. The strength of the binding of these metabolic intermediates varies depending upon the nature of the ligand, and therefore the classification of this interaction as reversible or irreversible is to some extent arbitrary. However, since the fundamental structure of the enzyme is unaltered and in most cases studied, displacing agents have been found which restore the original activity of the cytochrome, the metabolic-intermediate complexes can be viewed as an additional class of reversible non-competitive inhibitors.

A number of methylenedioxyaryl insecticide synergists (e.g. piperonyl butoxide) act as non-competitive inhibitors of the liver microsomal mixed function oxidases by forming such stable metabolic-intermediate complexes with cytochrome P-450 following their metabolic activation to either carbenes or other reactive intermediates (Franklin, 1976). Many other compounds, such as amphetamines, nitroso derivatives, and many amines such as desipramine, methadone, and diphenhydramine, form stable nitrogen-liganded, metabolic-intermediate complexes with consequent non-competitive inhibition of cytochrome P-450 (Franklin, 1977). A similar complex resulting in the non-competitive inhibition of cytochrome P-450-mediated mixed function oxidation has been demonstrated for isoniazid (Muakkassah *et al.*, 1981).

Cytochrome P-450-mediated mixed function oxidation can also be inhibited, frequently with severe toxicological consequences, by compounds which result in the eventual destruction of the haemoprotein. Such a process can be considered as being irreversible, non-competitive inhibition. Although compounds are known which cause the destruction of cytochrome P-450 directly, the majority of compounds producing this effect require a prior metabolic activation. The activation process is usually a consequence of a reaction mediated by cytochrome P-450 such that the damaging chemical species is produced in proximity to its site of interaction and damage. These processes have been termed 'suicidal activation'. The activation process can be either oxidative or reductive and the initial site of attack on the cytochrome can be either the haem moiety or the apoprotein. Consequently, there is a variety of mechanisms by which the enzyme damage can occur, each being characteristic to a large extent of the particular substrate concerned. Although the suicidal activation may occur preferentially with one isoenzyme of cytochrome P-450, because of the overlap of substrate specificities between the various isoenzymes, sufficient exposure to such a toxin can result in loss of a high proportion of the total cytochrome P-450. In rats, a 0.4-ml oral dose of carbon tetrachloride caused approximately 70% loss of hepatic cytochrome P-450 24 hours later (Glende, 1972). One of the best known groups of suicidal

activators is the polyhalogenated alkanes. A reductive process is responsible for the activation of several of these, such as carbon tetrachloride (Ahr *et al.*, 1980) and halothane (Ahr *et al.*, 1982), resulting in the formation of a free radical which causes destruction by an attack on the haem structure (De Groot and Haas, 1981). A further group of intensively investigated suicidal activators is various alkene derivatives, including allyl compounds (allylisopropylacetamide and allylbarbiturates) and halogenoalkenes (vinyl chloride and trichloroethylene). The allyl derivatives require oxidative metabolism for activation but the expected epoxidation reaction is not fulfilled and haem *N*-alkylation occurs from a reaction intermediate, resulting in the destruction of the cytochrome (Ortiz de Montellano *et al.*, 1979). The mechanism by which the halogenoalkenes cause cytochrome P-450 destruction has not been clearly established, but an interesting feature is the selective destruction of a cytochrome P-450 isoenzyme while the isoenzyme, cytochrome P-448, is unaffected (Pessayre *et al.*, 1979; Reynolds *et al.*, 1975).

Sulphur-containing compounds, including the sulphur-containing organophosphates, have wide use as industrial compounds, pesticides, and pharmaceutical agents. As a result of cytochrome P-450-mediated oxidative metabolism, many of these compounds release atomic sulphur, sulphene, which interacts with cysteinyl and other amino acid residues in many cellular proteins. Destruction of cytochrome P-450 is a common effect of these compounds, the attack of the sulphene being at sites on the apoprotein (Kamataki and Neal, 1976). Many other compounds (Ortiz de Montellano and Correia, 1983), in addition to those discussed, caused marked destruction of cytochrome P-450 as a result of oxidative metabolism and, as a consequence, disturbances of haem metabolism such as porphyria frequently accompany this suicidal activation and destruction (Smith and De Matteis, 1980).

A reduction in the tissue content of cytochrome P-450 mixed function oxidases is also a feature of a number of metal ions, notably cobalt, cadmium, lead, copper, nickel, and manganese (Sasame and Boyd, 1978). These ions do not interact directly with the cytochrome but exert their effect both by inhibiting synthesis of the haem prosthetic group and by inducing the activity of the catabolic enzyme haem oxygenase. Metal ions may also affect other xenobiotic-metabolizing enzymes; for example, cadmium, mercury, and zinc non-competitively inhibit epoxide hydratase thereby potentiating the toxicity of those compounds which are oxidatively metabolized to reactive epoxides (Parkki, 1980).

In view of the destructive effect on the mixed function oxidase enzymes by these classes of non-competitive inhibitors, the impairment of metabolism will persist, generally for a longer period of time than that seen with the groups of reversible inhibitors, and the return of the metabolic activity to normal will be dependent on the rate of synthesis of new enzyme. In the case of exposure of rats to carbon tetrachloride cited earlier (Glende, 1972), more than seven days were required for the hepatic cytochrome P-450 content and its associated mixed function oxidase activity to return to normal values. The toxicological implications of exposure to

a mixture of substrates, in cases where such a significant reduction in metabolic activity occurs over such a time period, could be considerable. For compounds where elimination from the body is largely dependent upon the rate of metabolism, a 50% decrease in metabolic activity will double the half-life; a 75% decrease in metabolic activity will quadruple the half-life, provided that the remaining amount of enzyme is still sufficient for metabolism to take place by first-order kinetics.

In extreme cases of high exposure to a mixture of substrates or when there is inadequate enzyme activity because of enzymic destruction, the rate of metabolism of a compound approaches zero order. This may arise because the enzyme becomes saturated or because the availability of an essential endogenous cofactor becomes rate limiting. An example of this is seen in paracetamol (acetaminophen) overdosage where the availability of sulphate for conjugation is limited and possibly glucuronide conjugation is approaching limitation (Slattery and Levy, 1979). In such cases, the continued exposure to toxic compounds results in dramatic increases in blood and tissue concentration of the xenobiotic, with severe toxic consequences.

A feature of xenobiotic metabolism is the existence of alternative pathways of metabolism. At low dosage the detoxicating pathways of metabolism appear to predominate, but these may be inhibited by large amounts of the compound, or by the simultaneous administration of other compounds. The saturation of the detoxicating pathways consequently shunts the chemical into the alternative pathways which may be more concerned with activation. For example, paracetamol is normally non-toxic, because in the small amounts that are usually administered it is rapidly conjugated with sulphuric and glucuronic acids, and these are excreted in the urine. The saturation of these detoxication pathways exposes the paracetamol to mixed function oxygenation, either in the aromatic ring (epoxide formation) or at the nitrogen atom (*N*-hydroxylation), both of which give rise to highly reactive intermediates. The prior, or simultaneous, administration of large amounts of drugs and other compounds which are metabolized predominantly by glucuronylation [e.g. camphor, carbenoxolone, morphine, chloramphenicol, and tetracycline (Bolanowska and Gessner, 1978)] would greatly enhance the production of the reactive intermediates. These reactive intermediates are detoxified by conjugation with glutathione and, in turn, sufficient demand upon this metabolic pathway causes it to become rate limited by the biosynthesis of glutathione. When this stage is reached, the detoxication resources of the cell are exhausted and the accumulated reactive intermediates express their hepatotoxicity (Davis *et al.*, 1974). Other components of a mixture may cause inhibition of the glutathione protective mechanism. For example, mercury inhibits glutathione reductase, glutathione synthetase, γ-glutamyltranspeptidase, and glutathione peroxidase, and would therefore be likely to enhance the toxicity and carcinogenicity of those chemicals which would normally be detoxicated by interaction with glutathione, such as paracetamol reactive

intermediates, halogenated alkane and aromatic hydrocarbons, and polycyclic hydrocarbons (Shung *et al.*, 1982).

As has been noted, the inhibition or saturation of the detoxication pathways of metabolism frequently results in the production of reactive intermediates in alternative pathways. Although in many instances these reactive intermediates cause toxicity by their own characteristic mechanism, evidence is accumulating to suggest that a number of them share the common property of generating reactive forms of oxygen. Inhibition of some of the enzymes of xenobiotic metabolism should certainly not be viewed as the only mechanism by which increased amounts of reactive oxygen species are produced, but it may be an important one in the case of response to a mixture of xenobiotics.

Reactive oxygen species, such as the hydroxyl radical, which results in lipid peroxidation, destruction of cellular membranes, depletion of cytochrome P-450, and cellular necrosis, may be generated from the reactive intermediates of certain compounds such as quinones formed by the mixed function oxidase activation of aromatic compounds, such as benzene, and certain drugs such as adriamycin (doxorubicin). These free hydroxyl radicals are generated from a cyclic process in which the quinones are reduced to semiquinones by cytosolic flavoprotein oxidoreductases and subsequently re-oxidized by oxygen. More recent studies indicate that non-haem iron (Fe^{2+}) is also necessary for the eventual production of hydroxyl radicals. These reactive oxygen radicals may also oxygenate non-enzymically xenobiotic molecules in sterically hindered positions, and thereby increase the rate of formation of reactive intermediates. Hence the simultaneous ingestion of chemicals such as benzene which can give rise to quinone formation may, in the presence of polycyclic aromatic carcinogens, lead to greater activation of the carcinogens, and to synergism of carcinogenesis.

Trichloroethylene has been shown to potentiate the hepatotoxicity of low doses of carbon tetrachloride; trichloroethylene *in vitro* did not produce lipid peroxidation but potentiated that initiated by carbon tetrachloride, causing subsequent decreases in tissue glutathione and microsomal cytochrome P-450 (see Table 6) (Pessayre *et al.*, 1982). Similarly, hexachlorobenzene increases the iron-induced mitochondrial lipid peroxidation and decreases mitochondrial function, probably because of the hexachlorobenzene induction of porphyrin accumulation (Hanstein *et al.*, 1981).

Similar effects may be anticipated from the co-oxygenation of xenobiotics during prostaglandin synthesis. In the formation of prostaglandins, the hydroperoxide intermediates (HEPTE) are converted to the corresponding hydroxy intermediates, with the loss of oxygen, and the simultaneous oxygenation of many xenobiotic compounds that happen to be present. It has now been shown that this co-oxygenation procedure results from the loss of oxygen from the HEPTE as a hydroxyl radical, which is then able to oxygenate carcinogens in sterically hindered positions, and consequently give rise to high yields of alkylated DNA products. The extent of DNA binding of benzo[*a*]pyrene, and

Table 6 Synergism of trichloroethylene on carbon tetrachloride hepatotoxicity in rats

Treatment	Hepatic necrosis histo-pathology (0–10)	Serum alanine amino-transferase (IU)	Hepatic cytochrome P-450 (nmol/mg protein)	Hepatic glutathione (μmol/g liver)
Control	0	30	0.9 ±0.1	4.9±0.2
Trichloroethylene (1 ml/kg)	0	45	0.85±0.1	5.1±0.2
Carbon tetrachloride (64 μl/kg)	2	30	0.9 ±0.1	5.3±0.3
Trichloroethylene (1 ml/kg) plus carbon tetrachloride (64 μl/kg)	9	640	0.4 ±0.05	3.9±0.3
Carbon tetrachloride (1 ml/kg)	8	480	0.35±0.1	5.6±0.4

Data from Pessayre *et al.* (1982).

other carcinogens following co-oxygenation during prostaglandin synthesis, is many times greater than it is with the normal cytochrome P-450-mediated oxygenation by liver homogenates. Consequently, any situation that gives rise to the formation of large amounts of prostaglandins, or to the release of large amounts of arachidonic acid from microsomal membranes, such as prolonged immune response, hyperimmune states, etc., may simultaneously result in the enhanced toxicity of xenobiotic compounds present, or the greater activation of ingested carcinogens.

5 ENZYME INDUCTION

The treatment of animals with a variety of xenobiotic compounds has been shown to result in the increased or decreased toxicity of a test xenobiotic, according to the various time intervals between exposure to the first compound and exposure to the test compound. Investigation of the rates of elimination of the test compound frequently shows it to be decreased in comparison with controls during the first 24 hours after administration of the first compound and progressively increased thereafter up to a maximum rate at around three days. In many cases, these effects are due to interaction with the cytochrome P-450-dependent mixed function oxidases, particularly for those lipophilic compounds where the rate of metabolism is the major factor in the rate of elimination. The initial phase of decreased rate of elimination is attributable to a decreased rate of metabolism due to enzyme inhibition as described in the previous section. The subsequent phase of increased rate of metabolism is due to a net increase in the

amount of enzyme, enzyme induction. This phenomenon occurs with many enzymes and is well recognized as a normal mechanism of regulation of the activity of enzymes of intermediary metabolism. The induction of xenobiotic-metabolizing enzymes, a particular feature of the cytochrome P-450 haemoproteins, may therefore be regarded as a normal biological response to a particular metabolic demand. The decrease in the plasma half-life of warfarin in man produced by daily doses of various sedative–hypnotic drugs taken for three weeks previously is shown in Table 7 (Macdonald *et al.*, 1969). The consequences of induction of the cytochrome P-450 enzymes, however, may be very different from that of an enzyme of normal intermediary metabolism. Whereas the latter normally act on a single substrate, the cytochrome P-450 enzymes accept almost any lipophilic, xenobiotic compounds as substrates, as well as a number of endogenous lipophilic compounds, such as steroids. Thus the enzyme induction produced in response to one particular compound may have far-reaching effects on the metabolism of other compounds with a variety of toxicological consequences.

Many different xenobiotic compounds, of diverse structure, may result in enzyme induction, the major requirement for this effect being that the inducing agent is a substrate of the cytochrome P-450-dependent mixed function oxidases, and that it is only slowly metabolized, so that it is attached to the binding site of the enzyme for long periods and decreases the amount of active enzyme available (Parke, 1975). The early stage of enzyme *inhibition* lasts for some 1–12 hours, and is followed by increased *de novo* synthesis of the enzyme, and increased enzymic activity characteristic of enzyme *induction*. In some instances, the rate of degradation of the enzyme is decreased, thereby enhancing the inductive effect. This 'substrate-type' induction of the drug-metabolizing enzymes of the liver, gastrointestinal tract and other tissues has been well characterized by the use of a wide variety of drugs (phenobarbitone, phenytoin, imipramine), food additives (butylated hydroxytoluene), pesticides (DDT, dieldrin) and various environmental chemicals (chlorinated biphenyls, etc.) (Parke, 1975).

Table 7 Decrease in the plasma half-life of warfarin in man produced by sedative–hypnotic drugs

Inducing agent	Daily dose (g)	Warfarin plasma half-life (h)
None	—	52.4 ± 6.2
Chloral betaine	1.74	38.2 ± 3.8
Phenobarbitone	0.12	29.1 ± 3.1
Glutathimide	1.0	28.5 ± 2.9

The inducing agent was administered daily for three weeks. The results are means ± SEM in a randomized cross-over study with healthy subjects. Data taken from Parke (1975).

Since the cytochrome P-450 mixed function oxidases exist in a number of different isoenzymic forms, an inducing agent would be expected to induce the synthesis of that form (or forms) which is best able to metabolize it. This has been found to be reasonably valid, and, consequently, inducing agents have been classified according to the form of the haemoprotein that they induce, for example the phenobarbitone group (cytochrome P-450 forms) and the polycyclic hydrocarbon group (cytochrome P-448 forms). Compounds from within the same group will induce the metabolism of other compounds in that group but may not induce the metabolism of compounds in other groups. A good example of this is furnished by the cytochrome P-448-dependent de-ethylation of ethoxyresorufin and 2-hydroxylation of biphenyl, reactions which are largely dependent upon the cytochrome P-448 form of the enzyme which is induced by 3-methylcholanthrene (Table 8) (Burke and Mayer, 1975). This classification can only be a general guide because all of the isoenzymes of the cytochromes have yet to be characterized and there is still debate on their total number. Furthermore, some inducing agents, such as the polychlorinated biphenyls, induce forms of the cytochromes which fall into both the cytochrome P-450 group and the P-448 group. This is not surprising when it is remembered that a compound may be metabolized by alternative pathways, one of which may be cytochrome P-450-dependent and the other cytochrome P-448-dependent. An example of this is seen in Table 8 where biphenyl 2-hydroxylation is largely performed by the 3-methylcholanthrene-inducible cytochrome P-448 whereas the phenobarbitone-inducible cytochrome P-450 can only support biphenyl 4-hydroxylation. Even though there are severe limitations to this classification because of the exceptions and the subdivisions which appear to exist within the groups, the two groups of enzymes show such different metabolic characteristics that their distinction is of toxicological importance.

The cytochrome P-448 type of enzyme induction occurs with a variety of carcinogenic polycyclic aromatic hydrocarbons (Parke, 1975), other environmental chemicals such as tetrachlorodibenzodioxin (TCDD), and various

Table 8 Effect of inducing agents on the metabolism of ethoxyresorufin and biphenyl

	Enzymic activity of hepatic microsomes (nmol product/min/nmol cytochrome)		
Metabolic reaction	Control	PB (P-450)	3-MC (P-448)
Ethoxyresorufin *O*-de-ethylation	0.14	0.05	14.3
Biphenyl 2-hydroxylation	0	0.05	0.58
Biphenyl 4-hydroxylation	1.3	2.5	2.1

Rats were pretreated with corn oil (control), phenobarbitone (PB) or 3-methylcholanthrene (3-MC). Data taken from Burke and Mayer (1975).

polychlorinated biphenyls (Parkinson *et al.*, 1981), hexabromobiphenyl (a component of Firemaster) (Robertson *et al.*, 1981), and other toxic and carcinogenic compounds. This type of induction appears to differ from the classical substrate induction of microsomal enzymes by the phenobarbitone group in the following respects: (1) a different form of the cytochrome is produced, cytochrome P-448; (2) there is no simultaneous increase in cytochrome P-450 reductase; (3) there is no simultaneous proliferation of the endoplasmic reticulum; (4) a cytosolic receptor for the inducing agent has been identified, which is indicative of 'hormonal-type' induction; (5) cytochromes P-448 tend to exist as stable complexes containing the inducing agent, e.g. 3-methylcholanthrene, isosafrole, etc.; and (6) cytochrome P-448 but not cytochrome P-450 is inducible in the mammalian placenta.

It seems likely that the induction of cytochrome P-450 may generally be expected to enhance the detoxication of ingested chemicals, so that induction of this form of the cytochrome would generally result in lower toxicity and carcinogenicity. For example, treatment of rats and mice with *trans*-stilbene oxide leads to induction of specific forms of cytochrome P-450 that increase hydroxylation of benzo[*a*]pyrene and 7,12-dimethylbenzanthracene at the K region (a site of detoxication) and also increase the epoxide-detoxicating enzyme, epoxide hydratase; this results in a marked decrease in the covalent binding of these carcinogens to DNA (Lesca *et al.*, 1981). In contrast, induction of cytochrome P-448 forms by carcinogens, and other compounds of the necessary chemical structure and conformation, e.g. β-naphthoflavone and TCDD, is likely to result in the greater activation of toxic compounds and carcinogens, with a greater risk of chemical toxicity and carcinogenicity. Unfortunately, the field is highly complex; there are many exceptions, and, because of the interplay of metabolism with the disposition and excretion of the chemicals, findings in the whole animal do not always accord with those found *in vitro*. For example, although DDT treatment of mice results in a slightly greater enhancement of the hydrolytic metabolism (detoxication) of parathion than of the desulphuration pathway to paraoxon (activation) *in vitro*, there is an increase in toxicity *in vivo*; similarly, although chlordane equally enhances both pathways of metabolism of parathion, it does give some protection against the toxicity of the organophosphate insecticide (Table 9) (Chapman and Leibman, 1971). Furthermore, the site of organ toxicity may be altered, as in the case of the pulmonary toxin, 4-ipomeanol, in rats pretreated with phenobarbitone (cytochrome P-450) or 3-methylcholanthrene (cytochrome P-448); phenobarbitone increased the urinary excretion of detoxication products, decreased the covalent binding of 4-ipomeanol in both lung and liver, and decreased the acute lethality of the toxin with no signs of hepatotoxicity. In contrast, 3-methylcholanthrene had no effect on excretion, decreased the covalent binding in the lung but at the expense of increased covalent binding in the liver, and decreased the acute lethality (pulmonary), although this was at the cost of hepatotoxicity (Statham and Boyd, 1982).

Table 9 Effect of treatment with DDT, chlordane, or 3-methylcholanthrene on parathion toxicity in mice

Treatment	LD_{50} (mg/kg)	Metabolism *in vitro* to: Phosphothionate (nmol/h/g liver) (A)	Paraoxon (nmol/h/g liver) (B)	Ratio B/A
None	10	120	140	1.2
DDT (100 mg/kg per day for 3 days)	7	310	300	1.0
None	10	70	90	1.3
Chlordane (100 mg/kg per day for 3 days)	35	170	200	1.2
None	10	90	90	1.0
3-Methylcholanthrene (20 mg/kg per day for 4 days)	15	50	65	1.3

Data from Chapman and Leibman (1971).

Numerous experimental examples exist where induction of cytochrome P-450 forms confers protection against toxicity from simultaneous or subsequent exposure to other toxic compounds, but as expected this is not a consistent finding. Treatment of rats with phenobarbitone (phenobarbital) stimulates the oxidative metabolism of toluene and benzene, and also decreases their toxicity (Ikeda and Ohtsuji, 1971). Similarly, dieldrin and many other pesticides are potent inducers of the liver microsomal mixed function oxidases (Fabacher *et al.*, 1980), and DDT, dieldrin, and phenobarbital have been shown to increase markedly the oxidative and hydrolytic metabolism of various pesticides, resulting in decreased toxicity of Bidrin (dicrotophos), phosphamidon, malathion, and dicrotophos in mice, but increased toxicity of dimethoate (activated by oxidative metabolism) (Menzer, 1970; Tseng and Menzer, 1974). DDT and its metabolite DDE similarly decrease the lethality and adrenal necrosis in rats of 7,12-dimethylbenz[*a*]anthracene (Turusov and Chemeris, 1976). Chlordane pretreatment of rats increased the microsomal mixed function oxidation of the pesticides carbaryl and carbofuran, while methyl mercury hydroxide inhibited this metabolism; when chlordane and methyl mercury were administered simultaneously the opposing effects annulled each other and metabolism of the pesticides was the same as in control animals (Lucier *et al.*, 1972). The mixture of hydrocarbons present in petroleum also results in hepatic microsomal enzyme induction and enhances the oxidative metabolism of antipyrine in petrol station attendants, and the metabolism of antipyrine, aminopyrine, ethylmorphine, aniline and benzo[*a*]pyrene in rats (Harman *et al.*, 1981).

Chronic ethanol administration, at high dosage, also produces induction of the hepatic microsomal mixed function oxidases, epoxide hydratase and glutathione *S*-transferases, and an increase of intracellular glutathione (Hetu *et al.*, 1982), which may represent a protective response to an increased production of reactive intermediates of various environmental xenobiotics from the alcohol-induced novel form of cytochrome P-450 (Ingelman-Sundberg and Hagbjörk, 1982). Chronic administration of alcohol has been shown to increase the oxidative metabolism of the carcinogen, dimethylnitrosamine, in rats (Schwarz *et al.*, 1980), and changes the target organ for carcinogenesis in the mouse, favouring the development of olfactory neuroepitheliomas in preference to liver tumours (Griciute *et al.*, 1981).

The specific induction of cytochrome P-448 by carcinogenic polycyclic hydrocarbons and other toxic chemicals leads generally to a greater predominance of toxic pathways of metabolism. Cigarette smoke, which contains polycyclic hydrocarbons, leads to an increase in the metabolites of benzo[*a*]pyrene covalently bound to the nucleic acid in rat trachea (Simberg and Uotila, 1978) and to benzo[*a*]pyrene hydroxylase activity in human placenta (Kapitulnik *et al.*, 1976). Pretreatment of rats with 3-methylcholanthrene had little effect, or slightly increased the hepatotoxicity of the carcinogen aflatoxin, whereas pretreatment with phenobarbital prevented the hepatic necrosis (Mgbodile *et al.*, 1975).

From this discussion, it is apparent that enzyme induction, particularly of the cytochrome-dependent mixed function oxidase, is a common response to a sufficiently high dose of lipophilic substrates. The toxic consequences of the enzyme induction will be almost entirely dependent upon the toxic properties of the parent compounds and their metabolites together with the specific metabolic characteristics of the forms of the cytochrome induced.

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