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DETECTION AND SIGNIFICANCE OF ACTIVE METABOLITES OF AGROCHEMICALS AND RELATED XENOBIOTICS IN ANIMALS

(Technical Report)

Prepared for publication by

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Detection and significance of active metabolites of agrochemicals and related xenobiotics in animals (Technical Report)

Synopsis

The production of active metabolites from agrochemicals, particularly pesticides, is a common event and often determines the nature and extent of the toxicological responses observed in both target and non-target species. The various pathways of mammalian metabolism by which agrochemicals are converted to toxicologically more active metabolites are reviewed and illustrated with examples. Methods to detect, measure and study active metabolites are discussed. The relative importance of these pathways are compared in various test animals and humans and other factors influencing the pathways of production and destruction of active metabolites are described. It is concluded that improved approaches to risk assessment are needed for agrochemicals that, where appropriate, take into account the complexities of their activation reactions and of species differences in the generation and responses to active metabolites.

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ABBREVIATIONS

2-AAF; 2-acetylaminofluorene
AFB1; aflatoxin B1
ALT; alanine aminotransferase
ANTU; α-naphthylthiourea
AST; aspartate aminotransferase
ATPase; adenosine 5'-triphosphatase
cDNA; complementary DNA
CPIA; 2-(p-chlorophenyl)isovaleric acid
DCP: (cis)-1,3-dichloropropene
DDT: 1,1-bis(p-chlorophenyl)-2,2,2-trichloroethane
DDOH; 1,1-bis(p-chlorophenyl)ethanol
DMPO; 3,4-dihydro-2,2-dimethyl-2H-pyrrole N-oxide
DNA; deoxyribonucleic acid
EDB; ethylene dibromide (1,2-dibromoethane)
ESR; electron spin resonance
FAD; flavine-adenine dinucleotide
GABA; γ-aminobutyric acid
GC; gas chromatography
GSH; glutathione
HCBD; hexachlorobutadiene
HPLC; high performance liquid chromatography
MS; mass spectrometry
MTD; maximum tolerated dose
NADPH; reduced nicotinamide-adenine dinucleotide phosphate
NBQI: N-acetyl-p-benzoquinone imine
N-OHAAF; 2-(N-hydroxyacetamido)fluorene
OOS-Me(O); O,O,S-trimethylphosphorothioate
OOS-Me(S); O,O,S-trimethylphosphorodithioate
OPP-Na; sodium salt of o-phenylphenol
OSS-Me(O); O,S,S-trimethylphosphorodithioate
OSMeO$^-$; O,S-dimethylphosphorothioic acid
P-450; cytochrome P-450
PAPS; 3'-phosphoadenosine 5'-phosphosulfate
PBQ; phenylbenzoquinone
RNA; ribonucleic acid
UDP; uridine 5'-diphosphate
UDPGA; UDP-glucuronic acid

1. OBJECTIVE

The purpose of this report is to provide an overview of the nature and toxicological significance of active metabolites with special reference to pesticides and other agrochemicals. We have attempted to provide a broad range of examples of known bioactivation reactions, to describe the status of methods for detecting and identifying active metabolites, to outline some of the toxicological consequences of their production, and to discuss the implications of bioactivation for accurate risk assessment. It is intended that the report should be of assistance to scientists concerned with conducting metabolic studies of agrochemicals, to toxicologists attempting to understand toxic mechanisms, and to personnel of regulatory agencies who must interpret toxicological results in developing risk assessments.

2. INTRODUCTION

Most chemicals that are foreign to the normal biochemistry of the body (xenobiotics) are eliminated from the mammalian system more or less rapidly. Among these compounds are a large number of agrochemicals including pesticides and animal drugs. To facilitate the clearance of poorly-excretable lipophilic xenobiotics, most organisms have available a battery of enzymes that are specialized for the conversion of lipophilic materials to hydrophilic metabolites which can readily be eliminated by excretion in body wastes. The rate of elimination is a major factor governing the severity and duration of any toxic effects that may occur. However, the metabolism of xenobiotics is not always benign. For 40 years it has been known that some products of xenobiotic metabolism are more toxic than the parent compound. In many cases they are entirely responsible for any adverse effects that follow exposure. These products are termed active (or activated) metabolites and the metabolic process by which they are produced is called activation or bioactivation.

Pesticides are unusual among poisons in being valued for their toxic actions against certain organisms. Metabolic activation may be desirable if it occurs preferentially in the target species (pest) and slowly or not at all in non-target organisms since this leads to an elevated margin of safety. Pesticides that undergo metabolic activation in the target species before expressing their toxicity are termed "propesticides". These have been reviewed, with an emphasis on insecticides and their acute toxicity (refs. 1,2). However, metabolic activation is also responsible for many of the undesirable toxic effects, both chronic and acute, that may be attributable to pesticides and other xenobiotics e.g. the majority of compounds, including agrochemicals, currently regarded as carcinogens exert their effects through active metabolites (ref. 3). A number of examples of pesticide activation by oxidative reactions have been reviewed by Casida and Ruzo (ref. 4) and Segall (ref. 5).

A critical requirement for reliable risk assessment is an understanding of the metabolic fate of agrochemicals in experimental animals and its relationship to their metabolism in humans and other organisms to be protected. This information is needed for:

1. accurate extrapolation of toxicity observed in experimental settings at high doses to that occurring at much lower doses which are environmentally realistic but experimentally impractical.
2. extrapolating toxic effects from one species to another, and, particularly, from experimental animals to humans.
3. predicting interactions between compounds when the exposure is to complex mixtures, as is the common experience.
4. understanding how such variables as age, genetic and environmental effects may alter the toxicity of a compound.

The existence of possible thresholds of toxicity and significant non-linearities in dose-response relationships also may result from the events surrounding the production and destruction of active metabolites. If not considered during risk assessment, this may lead to significant inaccuracies.

Because of their critical importance in toxicology, the mechanisms and significance of xenobiotic activations have received considerable attention. A series of International Symposia on Biological Reactive Intermediates has been held and the proceedings published

(e.g. refs. 6,7). Aspects of the topic are regularly reviewed (refs. 3, 8-13). However, most of these reviews have considered agrochemicals only in passing, which does less than justice to their toxicological significance. Activation of these xenobiotics is frequently observed. Some occupational exposure is unavoidable and it may be excessive without the use of safety precautions. Further, the exposure of human populations to agrochemical residues in small amounts in food is virtually universal. Literature for this article was reviewed through June, 1992.

3. THE NATURE OF XENOBIOTIC ACTIVATION REACTIONS

Xenobiotic metabolism is generally regarded as occurring in two phases (Fig. 1). In Phase I, a reactive functional group is introduced into or revealed in the molecule. Most frequently this involves an oxidation reaction. Hydrolyses and reductions occur to a more limited extent. Phase I reactions are likely to be most significant with lipophilic xenobiotics that cannot be excreted efficiently without metabolic modification.

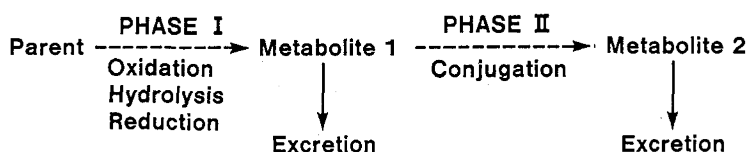


Fig. 1. The two phases of metabolism of most xenobiotics leading to metabolites that are excreted from the body.

In Phase II, excretable water soluble conjugates are formed by coupling such hydrophilic moieties as sulfate, glucuronic acid, water or glutathione to the reactive functional group introduced or revealed in Phase I. This biphasic scheme is common in the metabolism of agrochemicals, but not invariable. Xenobiotics that already possess reactive functional groups may bypass Phase I and be conjugated directly in Phase II reactions. Also, some products of Phase I, such as phosphoric acids from the hydrolysis of phosphate triesters, are sufficiently water soluble to be excreted directly without the intervention of Phase II conjugations. Although Phase I is associated with the broadest range of activation reactions, important activations may also occur through Phase II processes.

It is important to distinguish clearly between "reactive" and "active" metabolites. Increased chemical reactivity and increased biological activity (enhanced toxicological potency) are not synonymous. A metabolite may be chemically more reactive than its parent, but toxicologically less significant. Similarly, toxicologically significant metabolites are not necessarily more chemically reactive. Nevertheless chemical reactivity and toxicological activity are linked since highly reactive metabolites may attack vital sites on proteins, nucleic acids and lipids leading to a variety of cellular injuries.

Active metabolites vary widely in their chemical and biochemical stabilities. The stability of the metabolite is important in defining and limiting the location of toxic reactions within the body. Half lives of active metabolites under physiological conditions vary from milliseconds to several hours or days. Thus some metabolites may have such short half-lives that they never leave the immediate environment of the enzyme that produced them, whereas others are stable enough to leave the cell and organ where they are produced and exert their toxic effect at a remote site. Some may even be stable enough to be excreted. Guengerich and Liebler (ref. 9) provide a more detailed classification of reactive intermediates into seven categories according to their stability in biological systems.

All gradations of toxicity are seen among agrochemical metabolites ranging from those that have no discernible intrinsic toxicity, to those that are not much altered in toxicity compared to the parent, to those that are responsible for all the adverse effects observed. The term active metabolite here is taken, somewhat arbitrarily, to mean a metabolite that is substantially or entirely responsible for the biochemical actions that lead to a toxic outcome. Finally, cases exist where the active metabolite has a similar mode of action to the parent, but is more effective in causing injury (e.g. many anticholinesterases that are oxidatively activated), as well as situations where the parent causes one type of injury and the metabolite

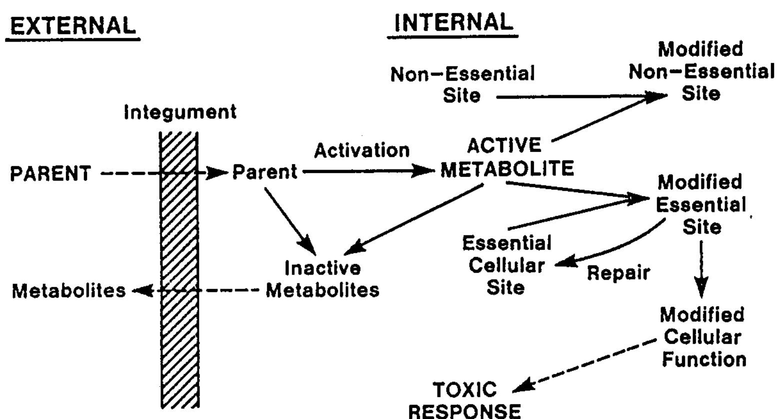


Fig. 2. Schematic representation of the general sequence of events leading to the generation of a toxic response from a compound requiring bioactivation.

has an entirely different one. The general relationship between activative metabolism and toxicity is illustrated in Fig. 2.

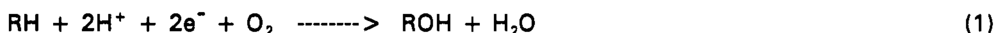
Although active metabolites may be produced from the parent in a single reaction, it is quite common for several successive enzymatic steps to occur before the final active compound is generated. For example, aromatic amines typically undergo N-hydroxylation and subsequent conjugation with sulfate or glucuronic acid before spontaneously forming nitrenium ions that can react covalently with cellular nucleic acids and proteins (ref. 18).

4. BIOCHEMICAL MECHANISMS BY WHICH ACTIVE METABOLITES ARE CREATED AND DESTROYED

The general enzymatic mechanisms by which xenobiotics are metabolized, including aspects of activative metabolism, have been reviewed in detail elsewhere (refs. 19-21) and only a brief outline is presented here.

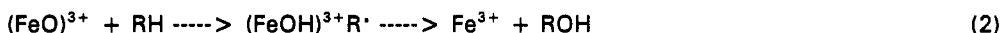
4.1 Reactions of Phase I

Cytochrome P-450-dependent monooxygenases. A majority of the instances of bioactivation are catalysed by a family of monooxygenase enzymes often termed mixed function oxidases (or monooxygenases) or polysubstrate monooxygenases in which the oxidation reaction is catalysed by the hemoprotein cytochrome P-450. Recent reviews of these oxygenases have been published focussing on vertebrates (ref. 22-26), insects (ref. 27), plants (ref. 28) and microbes (ref. 29). They are located primarily in the endoplasmic reticulum of many tissues with the liver of particular significance quantitatively. In this system, electrons from NADPH are delivered to cytochrome P-450 by the flavoprotein NADPH-cytochrome P-450 reductase. Cytochrome P-450 can then use molecular oxygen to oxidize a very broad range of xenobiotic substrates (RH) as shown in equation 1.



$$RH + 2H^{+} + 2e^{-} + O_2 \text{ -------->} ROH + H_2O \quad (1)$$

The reaction is thought to result from the action of a heme perferryl oxygen complex, $(FeO)^{3+}$, which inserts oxygen into the substrate through an intermediate radical complex (ref. 9):



$$(FeO)^{3+} + RH \text{ ----->} (FeOH)^{3+}R\cdot \text{ ----->} Fe^{3+} + ROH \quad (2)$$

Multiple forms of cytochrome P-450 occur which are tissue, species, age, and sometimes sex-specific. Genes for 40 forms of P-450 have so far been distinguished in rat tissues and 32 human genes have been identified (ref. 30). They have been classified into several families based on gene homology. The substrate specificity of these isozymes may be quite narrow or rather broad and tends to be overlapping so that several isozymes may metabolize a given

xenobiotic in a single tissue. The large number and the broad substrate specificity of many of these forms makes the P-450 monooxygenase system very versatile and important in the metabolism of lipophilic xenobiotics. Considerable differences in the properties of P-450s exist between species. This leads to corresponding variations in metabolic capabilities which may be reflected in differential responses to toxicants (ref. 24, 31). A toxicologically important feature of the P-450 monooxygenase system is the rapid inducibility of new forms of the enzyme which occurs within a few hours after exposure to a range of exogenous chemicals (refs. 32-34), including many agrochemicals (ref. 35). The induction of P-450s may alter both the rate and route of metabolism of a compound under study with clear toxicological consequences.

Typical oxidative reactions catalysed by P-450 are:

1. Aliphatic and aromatic carbon hydroxylations
2. O-, S-, N-dealkylations
3. Epoxidation of double bonds, sometimes followed by rearrangement (e.g. the NIH shift)
4. Heteroatom (N, S, P) oxidations

Cytochrome P-450 is also capable of catalysing reductions through direct electron transfer to a suitable acceptor substrate which in essence competes with oxygen for the electrons (ref. 9, 36). These reactions occur most readily at low oxygen tensions. Reductions catalysed by P-450 include the conversion of quinones to semiquinone radicals, nitro groups to nitro-anion radicals, and carbon tetrachloride to the trichloromethyl radical. Evidence has also been presented for the further reduction of nitro groups and N-oxides to amines, arene epoxides to the corresponding aromatic moiety, sulfoxides to sulfides, and azo compounds to hydrazines.

Flavine-catalysed monooxygenases. Vertebrate tissues also contain NADPH-dependent flavine-catalysed monooxygenases with a fairly broad substrate range that are capable of oxidizing many xenobiotics (refs. 37, 38). These enzymes contain one molecule of FAD but lack cytochromes. They are particularly associated with oxidations of heteroatoms such as nitrogen, sulfur and trivalent phosphorus. Oxidations at carbon atoms are not generally observed. Their significant role in the metabolism of some pesticides has been reviewed (ref. 39). Since these enzymes are microsomally located, driven by NADPH, and produce metabolites that are often identical to those generated by P-450-dependent monooxygenases, the potential for confusion between the two systems is considerable, but the use of selective inhibitors and the relative thermal instability of the FAD enzyme allows them to be distinguished (ref. 40). Multiple forms of this enzyme probably exist, and although this has not been thoroughly explored, it is clear that considerable variations exist in flavine monooxygenases from different tissues (ref. 41). These enzymes are not known to occur in invertebrates or plants.

Other oxidizing enzymes. Several other oxidases may be involved in xenobiotic metabolism and activation on a more limited or occasional basis. These include prostaglandin synthase, peroxidases, monoamine oxidase, and dopamine β-hydroxylase. These oxidases may be most significant toxicologically in extrahepatic tissues that are comparatively low in cytochrome P-450 monooxygenases such as the bladder, skin or lung (ref. 42). Further detailed information on these reactions is provided by Guengerich (ref. 38) and Smith et al. (ref. 42; prostaglandin synthase).

Hydrolases. A wide variety of esterases exist in vertebrate tissues, e.g., mammals have about 10-15 carboxyesterase with differing substrate specificities. Liver membrane fractions provide the richest source (ref. 43). These and other esterases are capable of hydrolysing carboxylate, thiocarboxylate, carboxamide, phosphate and some other types of esters. They have been classified as A- (aromatic), B- (aliphatic) and C- (acetyl) esterases depending, in part, on their responses to organophosphate inhibitors (ref. 44). Esterases play an important role in the detoxicative metabolism of some pesticides such as pyrethroid and organophosphate insecticides (ref. 44). Cases in which esterase cleavage acts as an activation are relatively uncommon, but the release of dinitrophenols from their esterified precursors (refs. 1, 45) and the conversion of the organophosphate, butonate, to trichlorfon (ref. 1) are examples of activative ester cleavage. Since P-450-containing monooxygenases are capable of the oxidative cleavage of some carboxylate and phosphate esters, the discovery of ester cleavage products does not necessarily indicate simple esterase action.

Reductases. Reduction is a reaction that occurs with several agrochemicals or their metabolites leading to potentially bioactive products. In particular, aromatic nitro reduction yields hydroxylamines and amines that are to further conjugated and ultimately create mutagens and carcinogens. Redox cycling of nitroaromatics (such as nitrofurantoin and related antibiotics), quinonoid compounds (e.g. the activation products of acetaminophen or o-phenylphenol) or bipyridinium herbicides (e.g. paraquat) leads to the production of free radicals, various forms of activated oxygen and ultimately to toxic effects through oxidative stress (ref. 46).

The action of cytochrome P-450s as reducing enzymes has already been described. However, several other flavoprotein-containing enzymes such as NADPH-cytochrome P450 reductase, xanthine oxidase, aldehyde oxidase, DT diaphorase, and mitochondrial complex I can also catalyse xenobiotic reductions such as the reduction of nitro groups (e.g. see ref. 47). The gut microflora may be another important source of reduced metabolites (ref. 48). Several enzymes capable of reducing xenobiotics are reviewed in Vol. 1 of ref. 19.

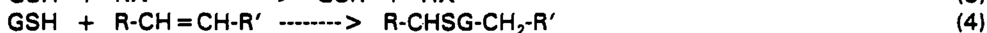
4.2 Reactions of Phase II

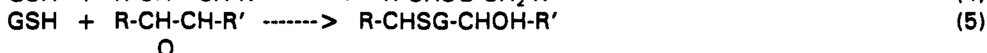
Phase II reactions are conjugations. The most important types in vertebrates are summarized below. Other, less common, conjugations also occur e.g. conjugation with glucose, a common reaction in insects and plants, also occurs in vertebrates but is relatively unimportant compared to glucuronic acid conjugations.

Glucuronidation. Glucuronidation, catalysed by glucuronyl (glucuronosyl) transferases using UDP-glucuronic acid (UDPGA), is a common reaction with phenolic and alcoholic metabolites of Phase I and is also seen with some xenobiotics containing carboxylic, thiol, and amino functions (refs. 49-51). Glucuronyl transferases represent a family of microsomally-located inducible enzymes with different but overlapping substrate specificities. Glucuronides are generally transported to the kidney where they are actively secreted. However, glucuronides with higher molecular weights are passed from the liver into the bile. Although glucuronidation operates primarily as a means of speeding the elimination of Phase I metabolites, several examples are known where it is an activating reaction particularly when glucuronidation of aromatic N-hydroxylamines or benzylic alcohols occurs. These yield, respectively, electrophilic nitrenium and carbenium ions after glucuronide group elimination. The reactivity of these glucuronides has been implicated in bladder and liver carcinogenicity. Some glucuronides are hydrolysed in the bladder by the acidity of the urine, or by bacterial glucuronidases in the gut, releasing the aglycone which may then exert its toxicity in that organ. The glucuronide thus has acted as a carrier for the ultimate carcinogen. Aglycones released in the gut after biliary excretion may also be reabsorbed into the circulation and return to the liver for reconjugation. This rather futile cyclical process is termed enterohepatic circulation.

Sulfate conjugation. This reaction parallels glucuronidation as a mechanism for enhancing the excretion of phenolic and other hydroxyl-containing xenobiotics (refs. 52, 53). Sulfotransferases utilize 3′-phosphoadenosine 5′-phosphosulfate (PAPS) as the source of the sulfate moiety. Sulfation has a rather low capacity *in vivo* and is readily "saturated" as the dose of xenobiotic is increased, leading to an increase in the glucuronide:sulfate conjugate ratio. In addition to phenols, sulfation may also occur with some anilines and other nitrogen-containing xenobiotics creating sulfamates. Sulfation typically eliminates biological activity and speeds urinary excretion. However, in a limited number of cases sulfate conjugates are highly reactive leading to cellular necrosis, and mutagenic and carcinogenic effects (ref. 54) e.g. the sulfate conjugate of 2-(N-hydroxyacetamido)fluorene (N-OHAAF) reacts even more readily than the corresponding glucuronide conjugate in covalent binding to proteins and nucleic acids. A similar situation is found with 1′-hydroxysafrole. As with glucuronides, the two types of sulfate conjugates most frequently involved in generating alkylating products are those of benzyl alcohols and aromatic N-hydroxylamines.

Glutathione conjugation. The tripeptide, glutathione (γ-glutamylcysteinylglycine; GSH), forms conjugates at the cysteine thiol group primarily with soft electrophiles (refs. 55-57). In addition to the nucleophilic displacement of leaving groups (eq. 3), addition of glutathione occurs across electrophilically-activated double bonds (eq. 4) and epoxides (eq. 5). Many such electrophiles exhibit a slow spontaneous reaction with GSH, but the rate is catalysed by a family of widely distributed and inducible GSH S-transferases.





Typically this is an important reaction that detoxifies reactive epoxides from Phase I and other environmentally-derived alkylating agents such as haloalkanes, haloalkenes and alkyl phosphate esters. More recently, it has become clear that GSH conjugation can also be an activating reaction in several different ways (refs. 12, 57). These include the direct generation of reactive intermediates such as a GSH episulfonium ion from the bifunctional alkylating agent, ethylene dibromide (EDB), or the release of cyanide from thiocyanates.

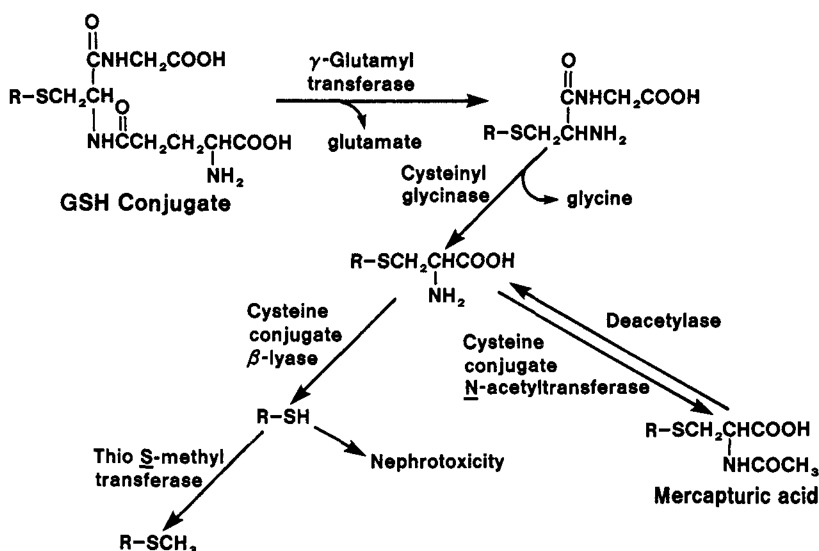


Fig. 3. Routes of further metabolism of glutathione conjugates to yield mercapturic acids, S-alkylthiols and reactive intermediates capable of causing nephrotoxicity.

A third mechanism of activation is through the production of conjugates of haloalkenes that are subject to further activative metabolism (Fig. 3). Glutathione conjugates typically are further converted to the corresponding cysteine conjugate which is then N-acetylated in the kidney to form a mercapturic acid as the final urinary excretory product. An alternative pathway of metabolism in the kidney is through a cysteine conjugate β-lyase (β-lyase) which cleaves the cysteine conjugate at the cysteine sulfur to release a thiol (RSH). Frequently these thiols are realkylated to relatively harmless products. However, as later examples will show, when the original GSH conjugate is with a haloalkene, this thiol is an α-chlorinated vinyl thiol. The thiol can rearrange to produce highly reactive metabolites (thioacyl halides or thioketenes) that cause nephrotoxicity and neoplasia (ref. 58).

Acetylation. The acetylation of amines, hydrazines, sulfonamides and hydroxylamines is an important Phase II reaction for a limited number of substrates (refs. 59, 60). Several examples of activation by acetylation are known. Generally the acetyl group donor is acetyl coenzyme A, but transacetylations e.g. from N-arylacetohydroxamic acids to arylamines or intramolecular acyl transfer by a distinct N,O-acetyltransferase have been observed (ref. 54). Examples of acetylation-related toxicity include kidney damage due to the acetylation of some sulfonamides yielding relatively insoluble conjugates that crystallize in the kidney tubule. Also, the acetylation of some aryl hydroxylamines, e.g. N-OHAAF, can produce alkylating agents in a comparable way to that seen with glucuronidation and sulfation.

Epoxide hydrolysis. Epoxide hydrolases, also termed epoxide hydratases or hydrases, catalyse the reaction of reactive epoxides with water to yield trans-1,2-dihydrodiols (ref. 61, 62). This is generally an important detoxication reaction. However, examples of activation through this mechanism are known e.g. epoxide hydrolase is often the rate limiting step in the formation of the diol epoxide mutagen/carcinogens derived from benz[a]pyrene and related polycyclic aromatic hydrocarbons (ref. 63).

Other conjugations. Other conjugations that may produce toxic products include the esterification of xenobiotic carboxylic acids that are produced by Phase I reactions (refs. 64, 65). Lipophilic carboxylic esters of this type can be formed with cholesterol or acylglycerols (R'OH in Fig. 4). Generally, but not invariably, the acid is converted to an intermediate coenzyme A derivative before esterification occurs. In a parallel fashion, a xenobiotic alcohol (R'OH in Fig. 4) may be conjugated with endogenous fatty acids to yield highly lipophilic and potentially toxic esters.

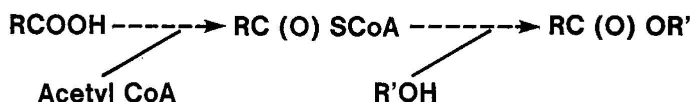


Fig. 4. General scheme for the conversion of xenobiotic acids (RCOOH) or alcohols (R'OH) to lipophilic esters.

Amino acid conjugates (e.g. with glycine or glutamine) are also formed from some xenobiotics containing carboxylate groups after they are converted to their coenzyme A derivatives, but there is little evidence, so far, that this leads to activations of toxicological significance (66).

5. ILLUSTRATIVE EXAMPLES OF ACTIVE METABOLITES

Pesticides are notable both for the frequency with which bioactivation occurs and the range of mechanisms involved. This may be largely due to the fact that commercial pesticides are selected for their high toxicity to specific life forms. As pointed out by Casida and Ruzo (ref. 4), this requirements is coupled with a need for safety to non-target organisms. Crops often must be protected from pests for several weeks yet environmental biodegradability is a key property. This demands a difficult blend of toxicity and safety and of stability and reactivity that is often best met with compounds that develop enhanced reactivity after entering biological systems. Here we have restricted ourselves to a number of examples to illustrate important principles and the range of mechanisms and toxic responses that can be seen with pesticide bioactivations. In a few cases, well-studied and instructive examples of the activation of other xenobiotics have been included.

5.1 Thiono-Sulfur Oxidations

An important activation reaction in pesticide chemistry is the conversion of phosphorothionate triesters to the corresponding phosphate triesters (Fig. 5, lower pathway; refs. 67, 68; reviewed in refs. 69, 70). This greatly enhances their ability to inhibit acetylcholinesterase in the nervous system and such compounds are very widely used as insecticides, acaricides and nematicides. Oxidative desulfuration is largely carried out by cytochrome P-450, although the flavine-dependent monooxygenase can also activate some phosphonothionates (ref. 71). The scheme below is thought to represent the pathway of the reaction which can also result in the dearylation of the triester (Fig. 5, upper pathway). Dearylation eliminates anticholinesterase activity.

The nature and fate of the sulfur atom released in this reaction was long ignored, but it, too, has toxicological significance since it is released in a thiol-reactive form that inhibits the P-450 system that produced it (refs. 72, 73). This causes differential inhibition of different isozymes of P-450 in the liver (ref. 74) and has the clear capability to alter the oxidation rate and thus the toxicology of both the parent compound and other xenobiotics to which the organism may be exposed.

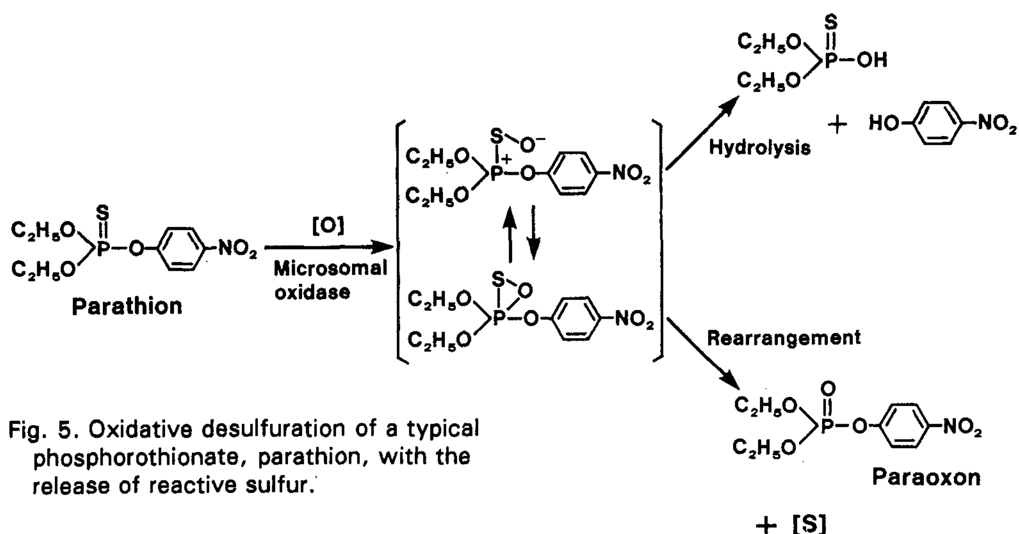


Fig. 5. Oxidative desulfuration of a typical phosphorothionate, parathion, with the release of reactive sulfur.

The ability of phosphorothionates to inhibit P-450 has been used commercially in the form of compound R-33865 (diethyl phenyl phosphorothionate; dietholate). This compound has been added to thiocarbamate herbicides as an "extender" to prevent too-rapid degradation in certain soils with high microbial activity. It is also an effective insecticide synergist (termed SV-1, ref. 75). R-33865 lacks appreciable anticholinesterase activity, but at high doses in mammals it causes liver necrosis and porphyria due to the high rate of clearance of P-450 heme damaged by the sulfur reaction. Pretreatment of rats with phenobarbital as an inducer of some P-450 catalysed oxidations results in enhanced liver damage from R-33865 (ref. 76).

A parallel situation exists with carbon disulfide which is widely used as a pesticidal fumigant. This too is metabolized by oxidative desulfuration to yield reactive sulfur and carbonyl sulfide, at the same time causing strong inhibition of P-450 and centrilobular degeneration of the liver. These effects also are potentiated by pretreatment of animals with the phenobarbital (ref. 70). In contrast, the rodenticide α-naphthylthiourea (ANTU) is primarily a pneumotoxin in rodents although persuasive evidence suggests that its toxicity also relates to the generation of reactive metabolite(s) through oxidative desulfuration (ref. 70). The corresponding urea is of much lower toxicity than its thiono analog which supports the idea that thiono-sulfur oxidation is the key event in the activation of ANTU.

Recently, a related type of oxidative desulfuration has been described with the new insecticide, diafenthiuron. This thiourea derivative is converted by sunlight and by oxidative metabolism to the corresponding carbodiimide (Fig. 6; refs. 77, 78). Since this carbodiimide is highly sterically hindered, it may have unusual stability. It irreversibly inhibits mitochondrial oxidative phosphorylation by reaction with the F_1F_0-ATPase (refs. 77, 78) an enzyme already known to be sensitive to carbodiimides. Specific binding to a mitochondrial 8 kDa F_0-ATPase proteolipid has been shown in rats. Additional binding to porin molecules has been observed in insect mitochondria (ref. 79).

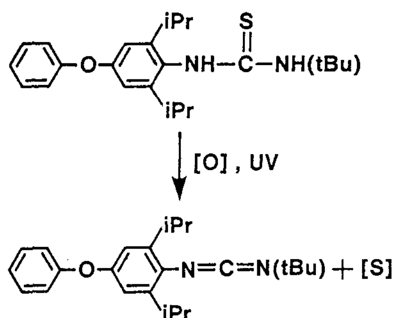


Fig. 6. Activation of diafenthiuron to a form a carbodiimide.

5.2 Thiolo-Sulfur Oxidations.

Phosphorothiolate esters. Some samples of organophosphate insecticides, particularly malathion, phenthoate and acephate, contain simple S-alkyl phosphorothiolate esters that cause a range of unexpected toxic effects, particularly in the lung. Typical examples are O,S,S-trimethyl phosphorodithioate [OSS-Me(O)] and O,O,S-trimethyl phosphorothioate [OOS-Me(O)] (ref. 80). At low doses, OSS-Me(O) displays toxicological properties similar to those of OOS-Me(O), but at higher doses cholinergic stimulation is also observed. Since neither of these compounds is a potent phosphorylating agent or inhibitor of acetylcholinesterase, it seemed probable that activation might be occurring *in vivo*. Studies with OOS-Me(O) confirm this (ref. 81). Further metabolic studies of OSS-Me(O) with lung and liver microsomes and slices (refs. 82, 83) showed that:

(1) The main metabolite (Fig. 7) is $OSMeO^-$; (2) the reaction is at least partly dependent on cytochrome P-450; (3) pulmonary microsomes are more active metabolically than hepatic ones; (4) protein-bound radioactivity is higher in lung slices than liver slices. The bioactivation of OSS-MeO therefore seems to involve sulfoxidation, rapid rearrangement to form a -P(O)-O-S- structure, and then release of $HOSCH_3$. This metabolite can form disulfides with proteins and GSH.

Interestingly, the toxicity of the phosphorothiolates is strongly antagonized by the presence of just a few percent of the phosphorothionate isomer of OSS-Me(O), O,O,S-trimethyl phosphorodithioate (OOS-Me(S)), in the mixture (ref. 80). Probably this can be explained by the inhibitory effect on sulfoxidation caused by sulfur release during P-450-catalysed desulfuration of the thiono isomer in a reaction comparable to those described with parathion and R-33865 (ref. 84).

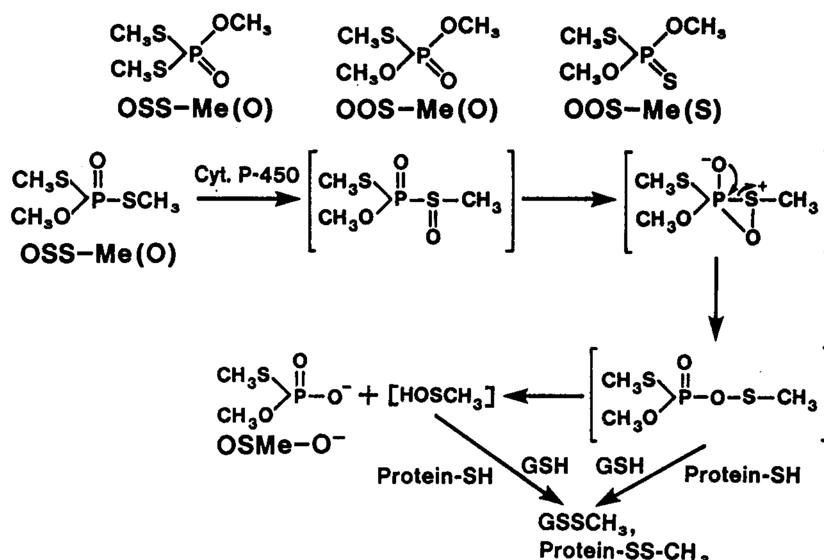


Fig. 7. Oxidative activation of the S-alkyl phosphorothiolate ester, O,S,S-trimethyl phosphorodithioate (OSS-Me(O)).

A similar mechanism of activation through sulfoxidation and rearrangement has been proposed for a number of widely-used S-alkyl (particularly S-propyl) phosphorothiolate insecticides that are cholinergic in their effects *in vivo* but lack strong anticholinesterase activity *in vitro*, e.g., profenophos and ethoprop (refs. 1, 4). Incubation of these compounds with active microsomes in the presence of acetylcholinesterase greatly enhances the inhibition of this enzyme, although the putative sulfoxide intermediate is too unstable to isolate. Other S-alkyl phosphorothiolates are used as defoliants (DEF; S,S,S-tributyl phosphorotrithioate) and as fungicides (e.g. Kitazin; O,O-diisopropyl S-benzyl phosphorothioate). Comparable sulfoxidation to more active phosphorylating agents probably occur in these cases also (ref. 4, 5).

Organophosphate insecticide thioethers. Activation of organophosphates by sulfoxidation is also commonly found in compounds containing thioether groups remote from the phosphorus center, e.g., disulfoton and fenthion (Fig. 8) and the related compounds, phorate and terbufos. In this case sulfoxide and sulfone formation yields metabolites with reasonable stability but with enhanced electron deficiency at the phosphorus center which leads to a considerable increase in ability to inhibit cholinesterase (ref. 1).

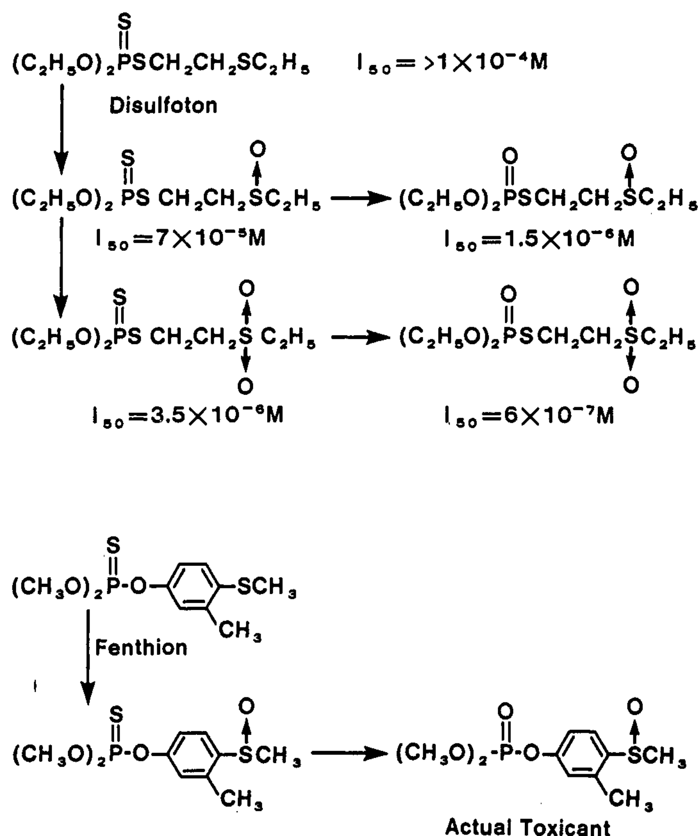


Fig. 8. Examples of the oxidative activation of the thioether-containing organophosphates, disulfoton and fenthion. I_{50} values are for the inhibition of acetylcholinesterase.

Sulfoxidation and sulfone formation in the dithiolane group are also critical for the anticholinesterase activity of the insecticides phosfolan and mephosfolan (ref. 4). Likewise, the oxime carbamate pesticide, aldicarb, is subject to rapid conversion to the sulfoxide and sulfone with increased anticholinesterase action (ref. 85).

Recent detailed studies with phorate (O,O-diethyl S-ethylthiomethyl phosphorodithioate) (refs. 40, 86) have revealed the complexity of its activation, which parallels that of disulfoton (Fig. 8). This consists of a combination of oxidative desulfuration at the phosphorus atom (purely a P-450 function) and successive thiol sulfur oxidations, which involves both P-450 and flavine monooxygenases. The relative activity of these two oxidative systems in producing sulfoxides varies from tissue to tissue and is also sex-dependant. The two types of microsomal oxidases even produce different enantiomers of phorate sulfoxide. Only P-450 is capable of catalysing the further oxidation of sulfoxides to sulfones at an appreciable rate. Since there is no close relationship between the ability of these oxidation products to inhibit acetylcholinesterase *in vitro* and their acute toxicity to rats, this study also illustrates a more general principle that it may be difficult to predict the exact relationship between activation and toxicity based solely on *in vitro* metabolism studies. *In vitro* biochemical potency is not always expressed *in vivo*.

Triazine herbicide thioethers. Several herbicidal triazines contain methylthio groups attached to the triazine ring that are readily converted to sulfoxides and sulfones.

This introduces a reactive center at the adjacent carbon and leads to arylation reactions with tissue thiol groups. The metabolically derived sulfoxides of s-triazines such as cyanatryn (ref. 87) and simetryn (ref. 88) have been shown to react covalently with hemoglobin. In a related case, the triazinone herbicide, metribuzin, is converted to a reactive intermediate that produces adducts in blood and liver and manifests toxicity through liver necrosis. This probably occurs after oxidative conversion to a sulfoxide that depletes glutathione and then alkylates tissue thiols (ref. 89).

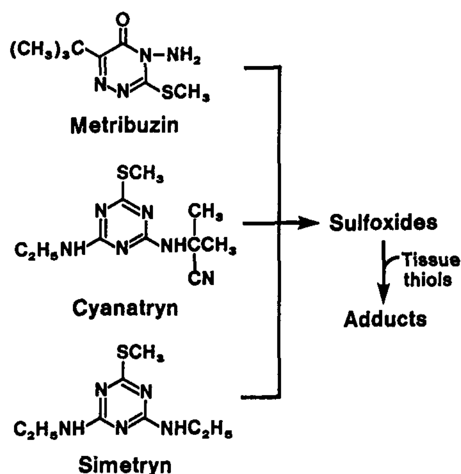


Fig. 9. Examples of triazine herbicides activated by sulfoxidation.

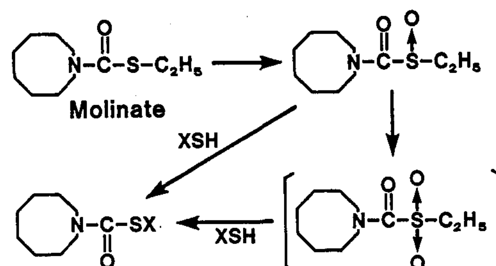


Fig. 10. Oxidative conversion of the thiocarbamate herbicide, molinate, to products with enhanced carbamoylating activity.

Thiocarbamate herbicides. A number of simple N,N,S-trialkyl thiocarbamates are widely used as herbicides e.g. EPTC (S-ethyl N,N-di-n-propylthiocarbamate) and its close relatives, butylate, molinate and thiobencarb. Studies of the metabolism of these compounds have revealed that sulfoxidation is a major reaction occurring in both plants and animals with possible further conversion to the corresponding sulfone (refs. 90, 91). The enzymes involved may be P-450s or, possibly, flavine-catalysed oxidases in vertebrates. The sulfoxide and sulfone analogs are considerably more active as carbamoylating agents than the parent and this may lead to a number of types of toxicity. Evidence suggests that these oxidation products are essential active metabolites in the phytotoxicity of thiocarbamates. Carp exposed to the rice herbicide, molinate, develop hemolytic anemia due to the reaction of the sulfoxide (and perhaps the sulfone) analog with hemoglobin (ref. 92).

The binding of thiocarbamates to hemoglobin has also been observed in mammalian metabolism studies. Reactivity towards GSH is enhanced by thiocarbamate sulfoxidation and this is a primary means for the detoxication of the active products.

A special situation following S-alkyl thiocarbamate sulfoxidation is seen with the herbicides sulfallate, diallate and triallate. Initially these compounds were discovered to be mutagenic in the Ames assay (ref. 93). This activity required the presence of a rat liver S-9 (microsomal) activating system. At first it was thought that hydrolysis occurred to release a 2-chloroallyl derivative which could undergo additional activation comparable to that seen with vinyl chloride. However, further study showed that the major activation occurs through sulfoxide and sulfone formation (Fig. 11).

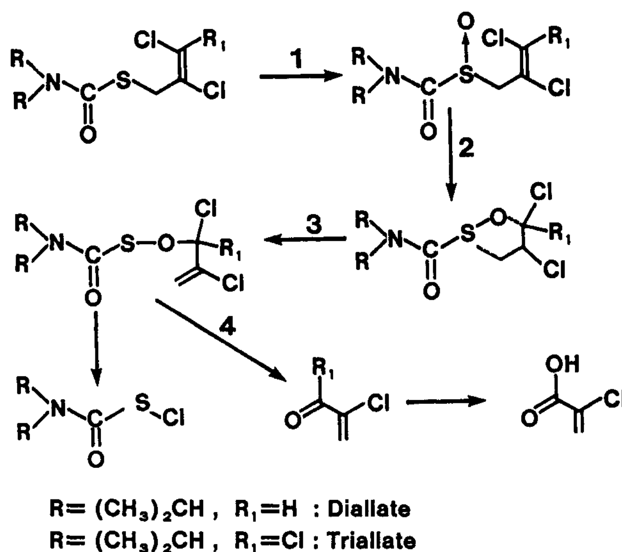


Fig. 11. Oxidative activation of the allylic thiocarbamate herbicides, diallate and triallate, to mutagenic metabolites.

The sulfoxide is mutagenic without further activation by a microsome-containing S-9 fraction (ref. 94). From studies with both biological activation and chemical oxidation with m-chloroperbenzoic acid, it was found that the initial formation of a sulfoxide (step 1) is followed by a [2,3]sigmatropic rearrangement (step 2) and 1,2-elimination (step 3) leading to the release of 2-chloroacrylaldehyde derivatives (step 4) that are powerful direct-acting mutagens (ref. 95). Critical structural features for this reaction have been defined (ref. 96). It is suggested that the genotoxicity of these herbicides results from the reaction of their reactive metabolites with purines, pyrimidines and their nucleosides to form fused-ring adducts. Carcinogenesis has been demonstrated with diallate in mice and sulfallate in rats (refs. 97, 98) but there are no reports of the carcinogenicity of triallate.

5.3 Oxidation of Dichloropropene Fumigants.

A second route to generating mutagenic 2-chloroacrylaldehydes has been observed with dichloropropenes that are widely used as soil fumigants (e.g. Telone(TM)). When freed of epoxides and other contaminants, (Z)(cis)-1,3-dichloropropene (DCP) is essentially inactive as a mutagen (ref. 99). Sequential conversion to the epoxide (1000), dichloropropanal (6,500) and 2-chloroacrylaldehyde (120,000) generates increasingly active mutagens (relative activity in the Ames Salmonella mutagenicity assay are in parentheses, DCP = 1) (ref. 100). All three active metabolites also occur as impurities in technical samples of the pesticidal mixture.

5.4 Oxidation of Acetaminophen and ortho-Phenylphenol.

Acetaminophen is not a pesticide but is very widely used as an analgesic drug. It provides a much-studied example of bioactivation with dual possibilities for causing tissue injury. Acetaminophen has been shown to cause hepatotoxicity when taken in overdoses, or at therapeutic doses in some special populations e.g., alcoholics. This drug is mainly eliminated as the sulfate and glucuronide conjugates. However, it is also converted by P-450 monooxygenases to a reactive electrophilic intermediate which is believed to be N-acetyl-p-benzoquinone imine (Fig. 12; refs. 9, 12).

As shown in Fig. 12, this activated metabolite can arylate cellular macromolecules, virtually exclusively at thiol groups, causing injury to tissues. Alternatively it can also accept an electron to form a free radical leading to cellular injury through oxidative stress (ref. 46). A high degree of protection against such attacks on vital sites is provided by the conjugation of this metabolite with GSH. However, this system has a finite capacity to remove the active metabolite. An overdose of acetaminophen causes depletion of hepatic GSH and finally the

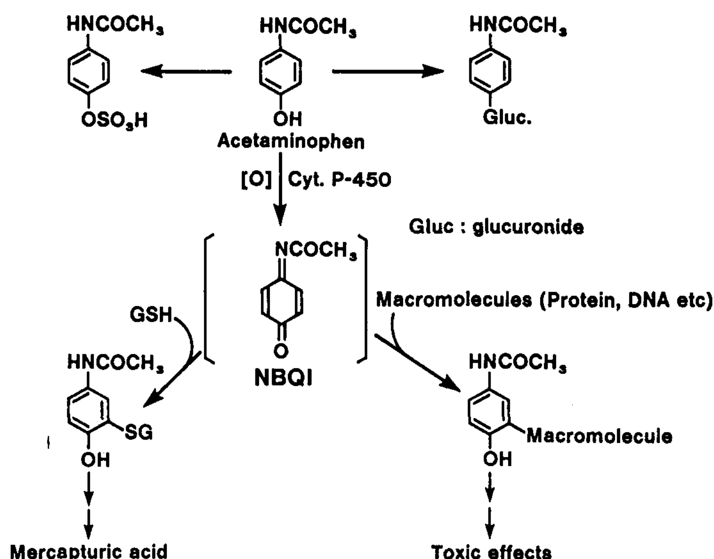
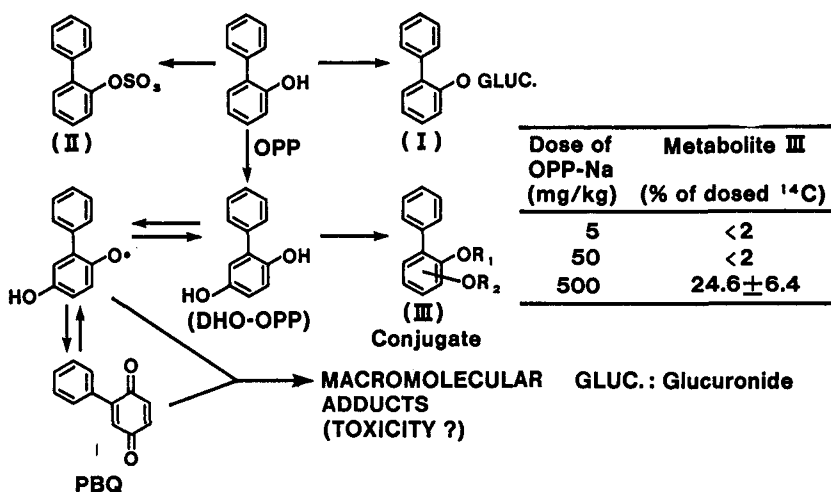


Fig. 12. Metabolic fate of acetaminophen including oxidative conversion to the reactive intermediate, N-acetyl-p-benzoquinone imine (NBQI).

essential sites are attacked leading to a cell death and a loss of hepatic function. Pretreatment with such compounds such as cysteine, methionine and GSH or their derivatives help to protect against the hepatotoxicity of overdoses by providing additional GSH and thiols in the liver. N-Acetylcysteine is used clinically for this purpose (ref. 101).

The fungicide o-phenylphenol and its sodium salt (OPP-Na) undergo a parallel reaction (Fig. 13) to produce the reactive quinonoid metabolite, phenylbenzoquinone (PBQ). This occurs after monooxygenase-catalysed ring hydroxylation and further oxidation, probably via a semiquinone free radical.



Dose of OPP-Na (mg/kg)	Metabolite III (% of dosed ^{14}C)
5	<2
50	<2
500	24.6 ± 6.4

Fig. 13. Metabolic fate of o-phenylphenol including its oxidative conversion to a reactive quinone (PBQ).

Phenylbenzoquinone is considered to the probable cause of the cytotoxicity and bladder and renal cancer associated with o-phenylphenol (ref. 102). Both arylation of tissue nucleophiles and redox cycling are again possible with this metabolite. Prostaglandin synthase may also be involved in the final production of PBQ in the bladder (ref. 103). Sulfation and glucuronidation are the primary metabolic reactions seen with o-phenylphenol at low doses with quinone formation occurring only at higher exposure levels as these reactions become capacity limited.

5.5 Generation of Mitochondrial Uncouplers by Oxidation or Hydrolysis

Mitochondrial uncouplers have varied uses as pesticides. However, their properties frequently must be modified to reduce toxicity to plants or vertebrates or for other reasons such as ease of formulation. Since most uncouplers are lipophilic weak acids, esterification or alkylation of the acid group has been a common mechanism of developing such propesticides. Examples where metabolism (N-dealkylation of amines or hydrolysis of acyl esters) releases the active uncoupler from an inactive precursor (Fig. 14) include the rodenticide bromethalin (ref. 104), a new perfluoroalkylsulfonamide insecticide, sulfluramid (ref. 105), the N-alkylated pyrrole insecticide, AC-303,630 (ref. 106), several types of esters of dinitrophenols (ref. 1, 45), and the trifluoromethylbenzimidazole acaricide, fenazaflor (ref. 107).

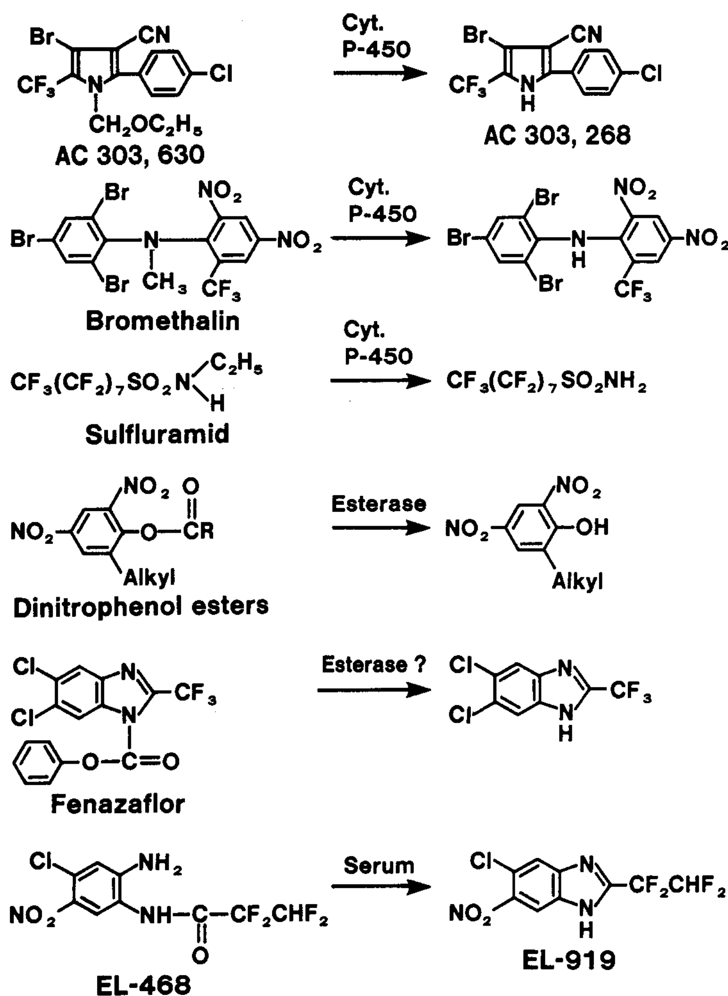


Fig. 14. Examples of the activation of pro-uncouplers by oxidative, hydrolytic or other mechanisms.

An unusual example of the generation of a powerful uncoupler *in vivo* from an inactive precursor is the serum-catalysed cyclization of the aminoanilide derivative, EL-468 (nifluridide) to its tetrafluoroethylbenzimidazole analog (EL-919) which has strong ectoparasiticidal action (ref. 108).

5.6 Activation by Reduction

Carbon tetrachloride. An important mechanism of metabolism resulting in tissue injury and possible carcinogenesis is the generation of free radicals and oxidative stress, as mentioned for the quinonoid metabolites of acetaminophen and o-phenylphenol. Another well-studied example is that of the fumigant additive, carbon tetrachloride. Carbon tetrachloride is converted to the trichloromethyl free radical by P450-catalysed reduction. A major result of free radical generation is lipid peroxidation and tissue thiol depletion. An alternative fate for this radical is oxidation by P-450 to yield phosgene which readily produces tissue adducts. The reduction of carbon tetrachloride is accompanied by the release of a reactive form of chlorine that can be trapped with 2,6-dimethylphenol to yield the 4-chloro analog (ref. 109). The complexities of the activation and toxic effects of carbon tetrachloride involving free radical generation, lipid peroxidation and disturbance of cellular calcium homeostasis are further reviewed by several authors (refs. 12, 110, 111).

Paraquat and related bipyridinium herbicides. The widely used herbicide paraquat and related bipyridinium herbicides depend on reduction and free radical production for their toxicity (Fig. 15).

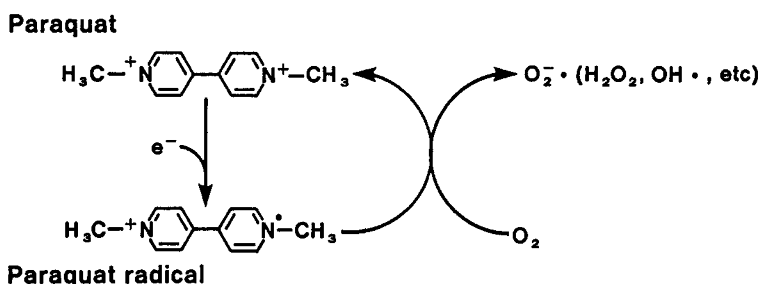


Fig. 15. Redox cycling of paraquat resulting in the generation of active oxygen species.

Paraquat is highly pneumotoxic due to its specific uptake and accumulation in the lung. Several electron transfer systems may supply electrons to paraquat, including the electron transport chains of photosynthesis, mitochondria and monooxygenases. Potentially toxic products resulting from paraquat's redox cycling and generation of reactive oxygen species include peroxides, superoxide and, particularly, hydroxide radicals (refs. 112, 113). These can account for its toxicity to both animals and plants.

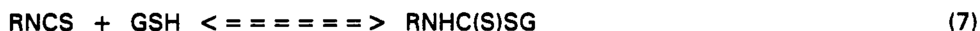
5.7 Glutathione Conjugations

At least three distinct pathways of activation by GSH conjugation are known with pesticidal compounds:

Reactions with thiocyanates and isothiocyanates. Alkyl thiocyanates react with GSH to release cyanide. Although a potential activation, it is not clear to what extent this reaction is involved in the acute toxicity of thiocyanates (e.g. Lethane insecticides) although toxicity does appear to parallel cyanide release (ref. 114).

RSCN + GSH ------> GSSR + HCN (6)

By contrast, isothiocyanates react with glutathione to produce dithiocarbamate esters. The reaction (eq. 7) is reversible and the intermediate isothiocyanate conjugate with GSH may be relatively stable (ref. 115).



RNCS + GSH < = = = = = = = > RNHC(S)SG (7)

The reversibility of this reaction can result in the transport of potentially toxic isothiocyanates as GSH conjugates widely through the body to be released subsequently in many tissues (ref. 115). A parallel reaction with methyl isocyanate may explain the unexpectedly widespread tissue damage observed in those exposed to this compound by inhalation during the Bhopal incident (ref. 116).

Formation of genotoxic agents from 1,2-dihaloalkanes. The fumigant, ethylene dibromide (1,2-dibromoethane; EDB) has been shown to be mutagenic and carcinogenic in a number of tests and species. The proximate active metabolite appears to be the episulfonium (thiiranium) ion formed by the reaction with GSH as shown in Fig. 16 (ref. 117).

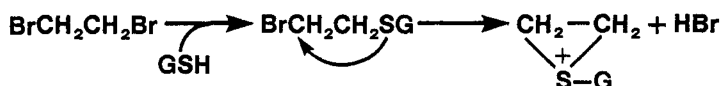


Fig. 16. Formation of a reactive episulfonium derivative by the reaction of EDB with glutathione.

Necessarily, only bifunctional alkylating agents are capable of this reaction. The fumigant 1,2-dibromo-3-chloropropane also is carcinogenic and follows a similar pathway of activation (ref. 118). Interestingly, the related fumigant, 1,2-dichloropropane appears to be activated by an oxidative pathway rather than by GSH conjugation (ref. 119) although alternative views have been expressed which implicate GSH conjugation as its primary activation route (ref. 12).

Glutathione conjugates that are activated by β-lyase action. Certain halogenated alkenes are nephrotoxic and carcinogenic because of initial conjugation with GSH and subsequent metabolism to release highly reactive products. The predominance of kidney pathology produced by such compounds arises because the final enzymatic conversion to reactive metabolites (β-lyase action) occurs primarily in this organ. The critical sequence of reactions is shown in Fig. 3. An example of nephrotoxicity arising from this mechanism is provided by the industrial intermediate and one-time pesticide (fungicide and herbicide) hexachlorobutadiene (HCBD; see Dekant et al. (ref. 120) for a review). β-Lyase action on the cysteine conjugate results in the release of a reactive thioacyl chloride and/or thioketene analog (refs. 121, 122). It is suggested that an early key event in the ultimate cytotoxicity of HCBD is the disruption of calcium homeostasis in mitochondria by the action of such products (ref. 121).

Tri- and tetrachloroethylene provide additional examples of activation by the combined action of several enzymes in the glutathione conjugation/β-lyase pathway. In this case, kidney cancer is seen in rodents only at very high doses where no more than a small percentage of the metabolites (less than 1% of the dose in the case of trichloroethylene) flows through this pathway (ref. 58). This illustrates an important point that relatively minor pathways metabolically may still be of great significance toxicologically. The identification of such "minor" pathways may be unusually challenging to the metabolic chemist but is critical for accurate risk assessment and management.

5.8 Sulfate and Related Conjugations

A well-studied example of activation by conjugation is the multiple- enzyme pathway by which 2-acetylaminofluorene (2-AAF) is converted to a highly reactive electrophile (Fig. 17; refs. 12, 123). While 2-AAF has never been used as a pesticide (fortunately it was rejected after serious consideration in this context some decades ago), the series of reactions it undergoes are typical of those of many other aromatic amines which are often metabolites of pesticides such as 4-chloro-o-toluidine, a metabolite of the insecticide/acaricide, chlordimeform. Such arylamines undergo N-hydroxylation to yield arylhydroxylamines (N-OHAAF in the case of 2-AAF). The hydroxyl group may then be conjugated with sulfate or glucuronic acid or acetylated. These derivatives, particularly the sulfate esters, are unstable leading to nitrenium ion formation and to covalent binding with nucleophilic groups of nucleic acids and proteins.

A second group of conjugates with similar instability is produced with benzylic alcohols. Well-known examples include 1'-hydroxysafrole and 1'-hydroxyestragole derived by monooxygenase action from the carcinogenic natural products, safrole and estragole. The sulfate esters of these alcohols are highly reactive via a putative carbenium ion intermediate that again forms covalent adducts with proteins and nucleic acids (ref. 12, 124).

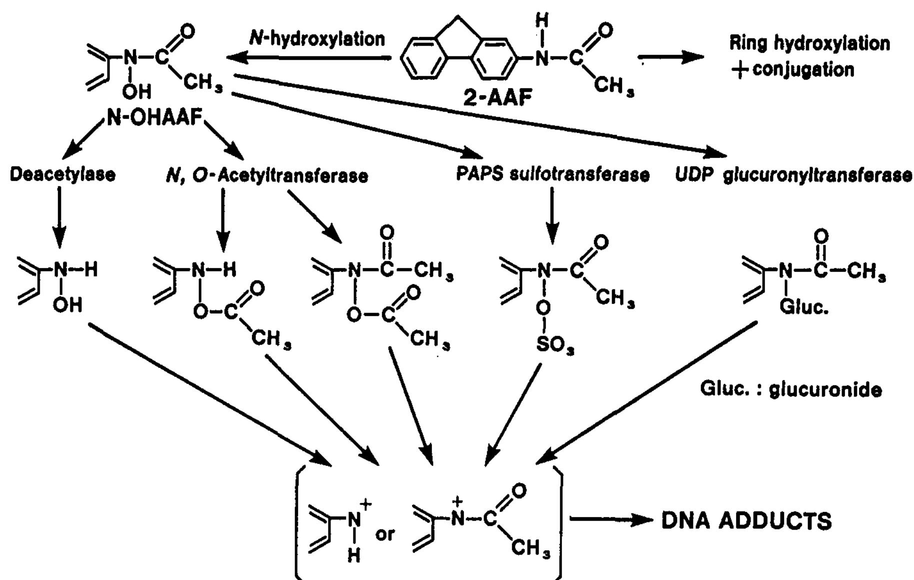


Fig. 17. *Multiple activation pathways for 2-acetylaminofluorene involving the formation of acetate, sulfate and glucuronide conjugates*

5.9 Lipophilic Carboxylate Esters

A rare type of activation occurs by the creation of lipophilic esters of xenobiotic carboxylic acids such as those formed by the hydrolysis of pyrethroid insecticides, aryloxyphenoxypropionate herbicides or the insect growth regulator, methoprene (ref. 65). These compounds may be conjugated with glycerol to form glycerides or with cholesterol to form cholesteryl esters. The resulting esters are highly lipophilic and may be stored in the tissues. They can, on occasion, cause unexpected toxicological responses.

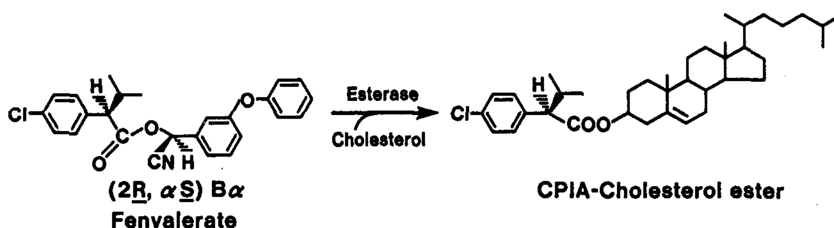


Fig. 18. *Production of a lipophilic cholesterol ester after esteratic hydrolysis of a fenvalerate enantiomer.*

For example, the ester pyrethroid, fenvalerate, at high doses produces fine needle-like inclusions that cause granulomas in several tissues (e.g. liver, spleen) of some species. The inclusions consist of the cholesteryl ester of the corresponding carboxylic acid (CPIA). Only the 2R,αS-isomer of fenvalerate causes this effect (ref. 125). This specific isomer is hydrolysed by a microsomal esterase. Transfer of the acyl residue to cholesterol occurs on the enzyme surface without release of the free acid (Fig. 18). The recent introduction of Asana(TM), a version of fenvalerate lacking the 2R,αS-fenvalerate isomer, avoids this problem.

Glycerol esters of such lipophilic acids have also been described (ref. 65). Diglycerides formed in this way could have tumor promoting properties akin to those of the phorbol esters which stimulate protein kinase C. Only weak activity was seen with a diglyceride containing the pyrethroid-derived 3-phenoxybenzoate group (ref. 126), but the discovery of even weak activity suggests that this mechanism of potential toxicity deserves further evaluation.

A further example is the biosynthesis of esters from cyclopropanecarboxylic acid, a metabolite of the acaricide, cycloprate (hexadecyl cyclopropanecarboxylate). The carnitine ester of cyclopropanecarboxylic acid is particularly interesting (Fig. 19).

It has been suggested (ref. 127) that the toxic effects associated with cycloprate relate to the sequestration of carnitine in this ester. The resulting carnitine deficiency leads to the inhibition of transport of fatty acids into mitochondria prior to β-oxidation.

A parallel biosynthesis of lipophilic esters can occur when naturally occurring fatty acids are conjugated with alcohols or phenols released from xenobiotics. Fatty acid conjugates of the mitochondrial uncoupler, pentachlorophenol, and the hydroxylated DDT metabolite, DDOH, are formed (refs. 128, 129) leading to prolonged retention in the body. These may have biological activity, e.g., the DDOH conjugate with palmitate has recently been shown to have behavioral effects and cause changes in brain muscarinic function in mice (ref. 130).

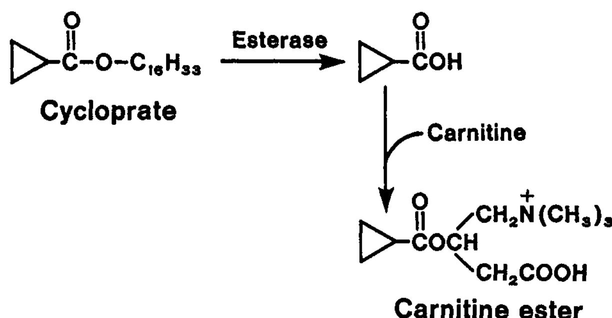


Fig. 19. Sequestration of carnitine by the hydrolysis product of the acaricide, cycloprate.

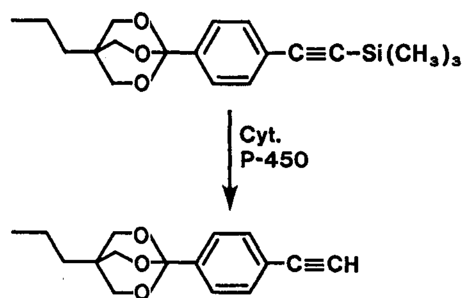


Fig. 20. Oxidative activation of an experimental silicon-containing insecticide to yield a neurotoxic GABA-antagonist.

5.10 Other Examples of the Metabolic Activation of Agrochemicals

Other instructive examples of the varied metabolic activation of pesticides include:

1. The conversion of captan and related N-haloalkylphthalimide fungicides to thiophosgene by reaction with tissue thiols (ref. 131) and possibly to other reactive species (ref. 132).
2. The generation of ethylenethiourea from ethylenebisdithiocarbamate fungicides and its further activation to potential carcinogens by both P-450 and flavoprotein-catalysed monooxygenases (ref. 133).
3. The hydrolytic release of unsymmetrical dimethylhydrazine from the plant growth regulator, daminozide, and its subsequent conversion to a DNA methylating agent (refs. 134, 135).
4. The hydrolytic release of 3,4-dichloroaniline from the herbicide propanil and its subsequent N-hydroxylation leading to methemoglobinemia and hemolytic anemia (refs. 136, 137).
5. The oxidative activation of the herbicides, dichlobenil (2,6-dichlorobenzonitrile) and chlorthiamid (2,6-dichlorothiobenzamide), to metabolites that binds covalently to macromolecules in the nasal mucosa causing necrosis (ref. 138).
6. Many examples of the activation of N-derivatized carbamate insecticides (e.g. N-sulfenylcarbamates) by reaction with tissue thiols or enzyme action to release active anticholinesterase agents (ref. 1).
7. The P-450-catalysed activation of a novel pro-insecticide probably involving oxidation at the silicon atom and yielding a potent and selective GABA-antagonist (Ref. 139; Fig. 20).

This listing does not exhaust known metabolic activations of agrochemicals. Further examples are provided in refs. 1, 2, 4 and 5.

6. DETECTION, IDENTIFICATION AND STUDY OF ACTIVE METABOLITES

6.1 Types of Evidence that Active Metabolites are Involved in a Toxic Response

There are several types of evidence that indicate the probable involvement of an activated metabolite in a toxic response:

1. Poor agreement between the observed toxic effect, the reactivity of the parent compound, and its *in vitro* activity on probable biochemical targets. Examples: the pneumotoxicity and other adverse effects of OOS-Me(O), the neurotoxicity of phosphorothionate anticholinesterases, and the acute toxicity of bromethalin, AC 303,630, and related pro-uncouplers (derivatized uncouplers that are activated *in vivo*).

2. Discovery of an active metabolite or a close analog as an impurity in technical samples of the parent agrochemical. In several cases these impurities have been shown to explain the biological activity of the parent *in vivo* or *in vitro*. Subsequent investigation has revealed the same compound as a metabolite. Examples: the estrogenic phenolic metabolites of methoxychlor, the epoxide and other mutagenic impurities in dichloropropene, and cholinesterase-inhibiting isomers and oxidation products of phosphorothionate insecticides.

3. Observation of the enhancement of the biological response by the addition of microsomal monooxygenases or other enzymes and their cofactors during *in vitro* test such as the Ames mutagenicity assay. Examples: the increased mutagenicity of diallate, related thiocarbamates and hexachlorobutadiene (HCBD), and the enhanced inhibitory activity of S-alkyl phosphorothioates in cholinesterase assays.

4. Discovery of the formation of adducts (bound residues) during metabolism studies either as unextractable tissue residues or by whole body autoradiography. Examples: EDB, cyantryn, HCBD and dichlobenil.

5. Identification and isolation of a reasonably stable active metabolite *in vitro* or *in vivo* during metabolism studies. Examples: thiocarbamate S-oxides or the desulfuration products of phosphorothionate triesters.

6. Identification of metabolites that are likely to have arisen from reactive intermediates. Examples: carbamoylated GSH from thiocarbamate S-oxides, or vicinal dihydrodiols and glutathione conjugates from reactive epoxides.

7. Effects of synergists or antagonists that act through metabolic enzymes on the toxicity of the parent compound. Examples: the antagonism of the toxicity of bromethalin by mixed function monooxygenase inhibitors, or the increased hepatotoxicity of carbon disulfide in animals pretreated with phenobarbital as a monooxygenase inducer, and the decreased toxicity of dinocap in animals pretreated with carbaryl which inhibits esterase-dependent activation. Negative results are also informative, e.g., Bowker and Casida (ref. 107) examined the activation of the pro-uncoupler, fenazaflor (Fig. 12), in mouse tissues. Neither carbaryl nor dichlorvos decreased the amount of activation in liver or serum suggesting that esterases are not involved in this heterocyclic carbamate cleavage reaction.

8. Observation that specific tissue injury occurs at a site remote from direct exposure to the parent compound. Frequently this may be related to specific activation reactions that occur in the remote tissue e.g. bladder cancer caused by some arylamines or kidney cancer caused by haloalkenes after conjugation with GSH.

Some of these types of observations may represent the first evidence of the existence of an activation reaction (e.g., examples 1, 4 and 8), while others are more likely to be used as confirmatory evidence that such products play an important role in the ultimate effects of the protoxicant (e.g., examples 5 and 7).

6.2 Methods to Study and Confirm the Role of Active Metabolites

Those metabolites with reasonable stability, e.g., paraoxon or thiocarbamate S-oxides or N-demethylbromethalin can be isolated, identified, and their fate and toxicology studied independently of the parent compound. Those that are much less stable present a considerable challenge to the analytical chemist and biochemist. In extreme, but not extraordinary cases, the active metabolite is too reactive to be identified directly or synthesized, and its production and effects can only be inferred from indirect evidence.

Isolation and synthesis. The simplest method to study and confirm the existence and action of an active metabolite that is reasonably stable chemically and biochemically (half life in tissues of at least a few minutes) is to isolate it from the system under study and/or to synthesize it. Many examples of this approach exist, e.g., general methods for the synthesis of xenobiotic conjugates have been reviewed (ref. 140). In some cases enzymatic synthesis has proved useful e.g. reactive aryl hydroxylamine conjugates have been prepared using microsomal glucuronyl transferase (ref. 141), and similar compounds have been isolated from the urine of treated animals and then used for further study (ref. 142). Special methods of extraction of labile conjugates may be necessary. For example, ion-pairing agents such as tetrabutylammonium bromide have been used to stabilize labile sulfate conjugates and allow their extraction with organic solvents (refs. 143, 144).

A common method in preparing and studying the chemistry and biological properties of many monooxygenase activation products is the use of biomimetic reactions involving organic peroxides, particularly m-chloroperbenzoic acid. The successes and limitations of this approach have been reviewed by Casida and Ruzo (ref. 4) and by Segall (ref. 5). Other biomimetic approaches to xenobiotic oxidations are discussed by Guengerich (ref. 38).

A more challenging situation is faced when the metabolite is too reactive to isolate and/or synthesize, e.g., it is estimated that the half life of the sulfate conjugate of N-OHAAF in water is about one minute (ref. 145) and other active metabolites react so rapidly that they interact primarily with the enzyme that produces them, e.g., suicide substrates such as the reactive sulfur released by P-450-catalysed oxidative desulfuration of thiono compounds. However, a variety of indirect methods described later provide evidence for the existence and nature of such reactive metabolites. The same indirect methods may be useful in studying more stable metabolites also.

In some cases there are advantages to presenting the prospective active metabolite or its precursor in a derivatized form from which it is readily released, e.g., the putative reactive thiol metabolites from β-lyase action (Fig. 3) may be too unstable to isolate but they can be studied indirectly in chemical and biological systems as their disulfides with 2-nitrothiophenol (refs. 146, 147). In studies of the GSH-dependent activation of EDB, van Balderen et al. (ref. 148) synthesized S-2-bromoethyl-N-acetylcysteine methyl ester as a model compound for the initial conjugate, S-2-bromoethylglutathione. This compound was highly mutagenic which supports the conclusion that cysteinyl conjugation is the major activating reaction for EDB's toxicity. Also, the dialkylcarbamoyl analogs of pyrrolizidine alkaloids have been used in studying the oxidative activation and toxicity of these natural hepatotoxicants (ref. 149).

Discovery and identification of covalent adducts. Many active metabolites are electrophiles that readily react with nucleophilic centers of tissue macromolecules. These adducts may be detected and quantified by using radiolabeled parents. Other methods of detecting conjugates include fluorometry, mass spectrometry (MS), immunology and ^{32}P-post-labeling techniques (refs. 150-152). These methods vary in sensitivity and their ability to provide structural information regarding the adduct. However, after the identification of the chemical structure of the bound residue, it may be possible to establish the nature of the original reactive intermediate.

The high sensitivity of some of these methods is indicated by the ability of an immunological assay for the 4-aminobiphenyl adduct of guanine to detect as few as two adducts/10^8 nucleotides (ref. 153). Current advances in MS allow similar or better sensitivity in some cases and ^{32}P-post-labeling may identify altered residues at as low a rate a 1 adduct in 10^{10}, though with no structural information. Recently Turteltaub et al. (ref. 154) have described

a method of DNA dosimetry based on accelerator mass spectroscopy with a sensitivity of 1 adduct per 10^{11} nucleotides and with the potential for a further increase in sensitivity of two orders of magnitude.

Trapping reactive metabolites. Reactive electrophilic metabolites may be trapped *in vitro* by adding simple nucleophiles to the reaction. For example, the highly reactive ultimate metabolites from GSH conjugation of HCBD were trapped with alkylamines and cyclopentadiene (ref. 121). Other reactive cysteine conjugate β-lyase products were trapped with benzyl bromide or diethylamine (ref. 58). Several investigators have used trapping agents to provide evidence for the formation of strongly electrophilic N-acetoxyarylamines by transacetylation reactions *in vitro*, e.g., by the conversion of N-acetylmethionine to yield methylthio-aminoarenes that can be identified by chromatography and MS (ref. 155-157). Guanosine has also been used as a trapping agent for these metabolites (ref. 156). Methanethiol was used to trap vinyl glyoxylate as a reactive product formed from homocysteinyl conjugates (ref. 158) and chlorine released during the reduction of carbon tetrachloride was revealed through reaction with 2,6-dimethylphenol to form the 4-chloro analog (ref. 109).

Water and GSH acts as natural trapping agent for many active metabolites and the identification of vicinal diol hydrolysis products or comparable GSH conjugates from arenes or alkenes is a reasonable indicator that a reactive epoxide intermediate has been produced. Similarly the structures of adducts formed in tissues such as those between phosgene and cysteine (ref. 159), the adducts of acetaminophen with tissue thiols (ref. 9), and the products of the reaction of 2-AAF (ref. 54) or EDB (ref. 160) with guanine residues from nucleic acids have been identified. Structural characterization of these adducts helps to indicate the nature of the active metabolites that produced them. Dimedone (ref. 161) or hydroxylamine (ref. 105) have been used to detect and trap the aldehyde products of oxidative dealkylation reactions. A flow method to estimate the stability of and trap reactive metabolites (alkylating agents) using a thiol-Sepharose column has been described recently (ref. 162).
The generation of active metabolites can also be confirmed by using biological systems as the trapping agent as in the Ames *Salmonella* mutagenicity assay. The addition of a rat hepatic S-9 fraction to provide monooxygenase action is a standard practice to reveal any oxidative conversion to mutagens. More complex requirements for generating mutagens can be defined using the Ames assay, e.g., Vamvakas et al. (ref. 163) showed that the activation of HCBD to mutagens required both GSH and a kidney fraction containing enzymes that convert the GSH conjugate to reactive thioacylating agents via the corresponding cysteine conjugate and β-lyase activity. Monooxygenase additions were not found to be effective in this case, thus helping to define the activation mechanism. Acetylcholinesterase has been used as biological indicator to trap unstable phosphorylating agents produced from insecticidal S-alkyl phosphorothioates (ref. 164) and phosphoramidates (ref. 165) by P-450 mediated oxidation. Mitochondrial reactions have been shown to activate uncoupler precursors through the slow increase in uncoupling activity during mitochondrial incubations (ref. 105).

Detection and trapping of radicals. In many cases, radicals may be detected and identified by ESR spectroscopy as they are formed, although the sensitivity may not be high. Unstable radicals can be detected by using spin-trap reagents, e.g., 3-nitrosodurene has been used to identify the photoproduct from the action of sunlight on the pyrethroid, fenvalerate (ref. 166). Thus prepared, stable nitroxide radicals can be separated by HPLC and analysed by ESR as well as GC/MS. Another spin-trap agent, DMPO (3,4-dihydro-2,2-dimethyl-2H-pyrrole N-oxide), has been used for detecting reactive oxygen species in other circumstances (ref. 167). Although it has proved difficult to identify the trichloromethyl radical produced from carbon tetrachloride directly by ESR, spin-trapping followed by ESR spectroscopy has allowed its identification (ref. 110).

Alteration of pathways of metabolite synthesis and destruction. Treatments that change (decrease or increase) the activity of enzymes suspected to be generating active metabolites may yield useful indirect evidence for the existence, specificity and significance of these pathways. These changes may be accomplished in several ways: by inhibition of the enzyme, by increasing its activity through induction, or by increasing or decreasing co-substrates or essential co-factors. This approach has been reviewed, including limitations and pitfalls, and particularly the lack of specificity of some methods (ref. 9).

Cytochrome P-450-catalysed reactions may be inhibited *in vitro* or *in vivo*, by such agents as the insecticide synergist, piperonyl butoxide [1-(2-propylpiperonyl)-2,5-dioxanonane], or SKF 525-A (proadifen). Examples of the use of these and related inhibitors abound, e.g., sulfur release from carbon disulfide is decreased in animals pretreated with SKF 525-A and increased where P-450 activity had been elevated by pretreatment with phenobarbital (ref. 70). In the latter case frank hepatotoxicity was observed. These changes were accompanied by equivalent changes in the binding of ^{35}S to liver macromolecules when $C^{35}S_2$ was administered. This reasonably supports a mechanism whereby P-450 releases sulfur in a reactive form that then binds on and near its site of origin. However, not all such results are unequivocal and some may be misleading e.g. despite the well-established and crucial role of cytochrome P-450s in activating phosphorothionate triesters, inhibition of cytochrome P-450 with piperonyl butoxide *in vivo* often increases their acute toxicity (ref. 168), although the response varies from compound to compound and also with the time after pretreatment with the inhibitor (ref. 169). Furthermore, pretreatment with P-450 monooxygenase inducers such as DDT, aldrin or phenobarbital often decreases rather than increases their acute toxicity (refs. 168, 170). The pharmacokinetics in this case are complex since P-450 is involved in both activation and deactivation reactions (Fig. 5) so that P-450 inhibitors and inducers may shift the balance in either direction. Similarly, administration of piperonyl butoxide to rats *in vivo* decreases the lung toxicity of the rodenticide ANTU (α-naphthylthiourea), which, together with other evidence, suggests that it is activated by P-450. However, piperonyl butoxide enhances the toxicity of ANTU when administered to the isolated lung (ref. 171). The situation regarding the site and nature of activation is therefore confused. Further, it must be remembered that there are multiple forms of cytochrome P-450 and thay are not equally sensitive to inhibitors such as piperonyl butoxide and SKF 525-A (ref. 172). Finally, results may be confounded by the fact that P-450 inhibitors can later (24-48 hours) cause P-450 induction, giving rise to a biphasic inhibition-induction effect (ref. 169). This can, for example, explain the variable effects of piperonyl butoxide treatment on phosphorothionate insecticide toxicity in rats depending on the interval between the the two doses (ref. 169).

Inhibitors have also been used to confirm the significance of esterase activation in several cases, e.g., carbaryl antagonizes the acute mammalian toxicity of the esterified uncoupler, dinobuton (ref. 45). The granulomatous changes caused by lipid conjugate deposition in rodents exposed to fenvalerate were antagonized by treatment with tetraethyl pyrophosphate which inhibits the esteratic cleavage of this pyrethroid (ref. 173).

Sulfotransferase inhibitors such as pentachlorophenol (ref. 174), salicylamide (ref. 175), 2,6-dichloro-4-nitrophenol (ref. 176) and dehydroepiandrosterone sulfate (ref. 177) have been used to provide strong circumstantial evidence for activation by sulfate conjugation, e.g., pentachlorophenol greatly reduces the covalent binding of N-OHAAF (ref. 178) and safrole (ref. 174) to DNA. In another example, D-galactosamine has been used to inhibit UDP-glucuronosyl transferase activation reactions in rat hepatocytes (ref. 179) and valproate and substituted-ethyl UDP analogs have been used for this purpose in other studies (ref. 180).

Several inhibitors including acivicin, a γ-glutamyl transferase inhibitor, have been used to show that the conversion of GSH conjugates to their cysteine analogs is essential for toxicity mediated through cysteine conjugate β-lyase (ref. 181). As an example of influencing activation by altering enzyme co-substrates, the *in vivo* depletion of GSH by pretreatment with diethyl maleate decreased the binding of EDB to DNA in hepatocytes (ref. 182). Other studies have employed phorone and vinylidene dichloride to deplete tissue GSH (ref. 183). The modulation of epoxide metabolism can be achieved *in vivo* using trichloropropene oxide to inhibit microsomal epoxide hydrolases and 4-fluorochalcone oxide to inhibit the soluble hydrolase (ref. 184). In this study, diethyl maleate was also used to deplete tissue GSH and buthionine sulfoximine to inhibit its biosysnthesis in order to assess the role of GSH in removing metabolically-generated epoxides.

The use of enzyme inducers to provide evidence of activation is very common and has been discussed by Gillette (ref. 185). An example in addition to those quoted previously is the observation that the toxicity of acetaminophen is increased by pretreatment of mice and hamsters with 3-methylcholanthrene, which increases the activity of P-450I isoenzymes, but is decreased by phenobarbital pretreatment which enhances P-450IIB type cytochromes (ref. 24). The caveat concerning the complexity of the P-450 system and the specificity of inducers applies as fully here as in the use of inhibitors.

Comparable methods are commonly used in *in vitro* studies with tissue fractions and enzymes to establish the formation and nature of active metabolites. Simply leaving out necessary cofactors for activating enzymes such as NADPH for flavine- or P-450-catalysed oxidations, GSH for GSH conjugations, or PAPS for sulfations may reduce or eliminate activation as measured by active metabolite accumulation, tissue adduct formation, target enzyme inhibition or mutagenic potency. For example, PAPS was shown to be essential for the covalent binding of 1'- hydroxysafrole to DNA, RNA and protein *in vitro* (ref. 186) and UDP-glucuronic acid has a similar effect in incubations with several arylhydroxylamines (ref. 187). However, care must be taken in interpreting such results too broadly since it does not always follow that because a reaction occurs readily *in vitro* with added cofactors it will occur equally readily, or at all, *in vivo*, where cofactor levels differ and competitive reactions may occur.

The use of specific enzyme inhibitors, such as those already mentioned, is another obvious and widely used tool *in vitro*. However, additional approaches are possible *in vitro* that cannot be employed *in vivo*, e.g., the use of antibodies or other inhibitors with poor ability to penetrate tissues. For example, in order to distinguish the relative contributions of flavine- and P-450-catalysed sulfur oxidations of phorate in fractions from a single tissue, an antibody to NADPH-cytochrome P-450 reductase was used which selectively eliminated the P-450 reaction. In turn, the flavine-catalysed monooxygenase activity could be selectively eliminated by heat denaturation (ref. 40). Specific antibodies offer an excellent approach for defining the exact nature of the enzymes involved in activation reactions in complex enzyme mixtures. Studies with purified enzymes offer a complementary (if highly artificial) approach, as in the phorate example where purified P-450 and flavine monooxygenase were also utilized to examine their comparative roles in sulfur oxidations (ref. 86).

Use of deuterium labeling. The deuterium kinetic isotope effect has been used in several studies to reveal the existence and nature of activation pathways that may involve the cleavage of C-H bonds. If cleavage of a C-H bond is rate limiting in the reaction, a clear reduction in rate will be seen with the C-D analog. The hepatotoxicity of chloroform provides an example of the use of this effect to determine the route of activation. Chloroform might be activated oxidatively by P-450 to the reactive metabolite, phosgene, or by reduction through P-450 to the dichloromethyl radical. The former reaction involves C-H cleavage, the latter does not. The fact that deuteriated chloroform is considerably less toxic than its protonated form suggests that the oxidative reaction to phosgene is the critical activation. This conclusion is supported by the isolation of the 2-oxothiazolidine-4-carboxylate adduct of phosgene with cysteine in treated animals and the lack of tritium label in microsomal proteins adducts after treatment with tritiated chloroform (ref. 159).

In contrast, fully deuteriated EDB is more genotoxic than its undeuteriated analog. This has been explained by the suppression of oxidative (C-H cleaving) reactions (pathway A in Fig. 21) through deuteriation which results in a greater availability of EDB for the competing critical activation reaction with GSH in which the C-D bonds remain intact (pathway B in Fig. 21; refs. 188 and 189).

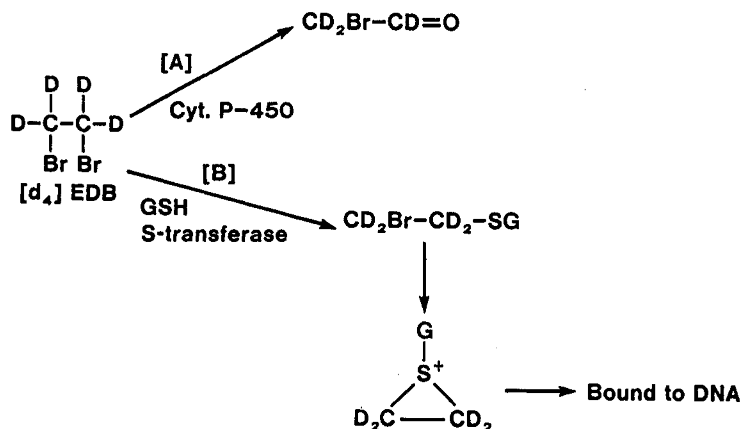


Fig. 21. Use of deuteriation to distinguish between alternative pathways for the metabolic activation of EDB.

The full retention of deuterium labeling in mercapturates produced after dosing animals with EDB or 1,3-dibromopropane also provides evidence in favor of the formation of a reactive episulfonium intermediate from these bifunctional alkylating agents. Recently it was shown that comparable studies with [$^2H_6$]-1,2-dichloropropane led to retention of only three deuteriums in the hydroxypropylmercapturate product, which suggests that an oxidative route is favored over episulfonium formation. This result may explain the much lower mutagenic/carcinogenic activity of 1,2-dichloropropane compared to the corresponding bromoalkanes (ref. 119)

Other examples in which deuteriated analogs provided useful information regarding activation include studies with 1,2-dimethylhydrazine (ref. 190), N-nitrosodimethylamine (ref. 191), cyclophosphamide (ref. 192), and proinsecticidal N-benzoyl-N-alkyl-2-aminothiazoles (ref. 193).

Activation in special species or strains of experimental organisms. Some strains or species of animals are known to be deficient in specific forms of xenobiotic metabolizing enzymes. These provide a useful means to demonstrate the significance of these enzymes in the toxicity of compounds, e.g., dogs, which lack N-acetyltransferase capacity, are resistant to the bladder carcinogenicity caused by N-arylamines in other species.

Another example is the use of brachymorphic mice with a much reduced ability to form PAPS and thus to generate sulfate conjugates. Boberg et al. (ref. 124) observed that in these mice there was much less binding of 1'-hydroxysafrole to DNA and RNA and a lower incidence of liver tumors compared to normal mice. Even differences between the sexes of the same species may be useful in exploring activation reactions, e.g., the lower sulfotransferase activity in female rat liver compared to that of males correlates with the decreased toxicity of N-OHAAF to female rats (ref. 194).

Table 1 lists some examples of species that are lacking in enzyme systems known to be involved in xenobiotic activations. It should be noted that in some cases the degree of "deficiency" depends on the substrate used.

TABLE 1. Some examples of deficiencies in xenobiotic metabolizing systems in vertebrate species.

REACTION	SPECIES SHOWING DEFICIENCY
Phase I:	
Aliphatic amine N-hydroxylation	Rat, marmoset
Arylacetamide N-hydroxylation	Guinea pig, steppe lemming
Phase II:	
Arylamine N-acetylation	Dog, fox
Glucuronidation (some substrates)	Cat, lion, lynx
	Squirrel monkey
	Rat (Gunn strain)
Sulfate conjugation	Pig, opossum
	Mouse (brachymorphic)
Glycine conjugation	African fruit bat, hen
Mercapturic acid synthesis	Guinea pig
Epoxide hydrolase	Rat (F344 strain)

Modified from Miyamoto et al. (ref. 31)

Microbial strains with specific metabolic deficiencies may also be useful indicators for activation mechanisms. The organophosphorus insecticide, fenitrothion, contains a nitrophenyl group. It causes an increase in mutations in the Ames Salmonella assay using strain TA100, but in a related strain (TA100 NR) that is deficient in nitroreductase activity, mutagenicity is eliminated and it is much reduced in a TA100 derivative stain lacking transacetylase activity (ref. 195). This indicates that, as with many nitroaromatics, the route to genotoxicity lies through an initial reduction of the nitro group and subsequent acetylation. Similarly, a *Salmonella* strain lacking acetyltransferase activity does not show the reversions typical of mutagenesis on exposure to aminofluorene (ref. 196).

Other special modified strains or surgical treatments of animals that alter metabolism can reveal critical information regarding activation. 2-Nitrotoluene induces unscheduled DNA repair (indicating damage to DNA) in male but not in female F344 rats. However DNA repair is not induced in germ-free male rats or in rats with cannulated bile ducts. This indicates that enterohepatic circulation and gut microflora are involved in the bioactivation that causes DNA damage. Male rats excreted 29% of a 200 mg/kg dose in the bile in 12 h compared to 10% for the female, and male rats showed a 3-fold higher binding of label to hepatic macromolecules than females. The critical bioactivation step probably involves reduction of the nitro group to an amino group and oxidation of the methyl to a hydroxymethyl group. Either of these intermediates may be subject to subsequent sulfation leading to reactive entities. As confirmatory evidence, it was found that sulfotransferase inhibitors also antagonize this toxicity (ref. 197).

Use of genetically modified cell lines. The cloning of genes for enzymes involved in xenobiotic metabolism, particularly cytochrome P450s, has allowed the development of novel cell lines in which the genes from one species may be transferred into cells from another or new combinations of metabolizing enzymes may be created. Recent results have been reviewed (refs. 198 and 199). In one example, a human lymphoblastoid cell line was metabolically augmented by transfection with cDNAs for cytochrome P450IIA2 and epoxide hydrolase. These cells were more sensitive to the mutagenic action of several types of promutagens, e.g., an elevated level of production of diols from benzo[a]pyrene was observed (ref. 200). Although such results are limited so far, transgenic cells will undoubtedly be useful in developing a more accurate picture of critical activation reactions catalysed by both animal and human enzymes. This is discussed in further detail in a recent review of the use of isolated cells in metabolic studies with agrochemicals (ref. 201.

7. THE SIGNIFICANCE OF ACTIVE METABOLITES

7.1 General Consequences of the Formation of Active Metabolite.

There are several possible biological consequences of active metabolite formation.

1. Non-covalent interactions occur when, compared to the parent, the metabolite better fits or activates a critical receptor or enzyme or is more active as an ionophore. Examples of this include the N-demethylation of formamidine insecticides leading to increased adrenergic receptor activity (ref. 202), the metabolic release of mitochondrial uncouplers from their derivatized propesticide forms (refs. 45, 104-106) and the O-demethylation of methoxychlor to enhance estrogenic activity (ref. 14).

2. Chemically reactive species are produced that covalently modify cellular proteins, nucleic acids and lipids. This may cause:

   a. Generalized cellular damage and necrosis.
   b. Specific physiological dysfunctions due to the irreversible inhibition of critical enzymes.
   c. Genetic damage leading to mutations, cancer or birth defects
   d. Immunotoxicity due to the antigenic reaction to a modified protein (e.g. see ref. 203).

3. Free radicals are produced, activated oxygen species are generated, and redox cycling occurs leading to oxidative stress (ref. 46) as outlined previously for compounds such as carbon tetrachloride, paraquat and o-phenylphenol.

It is very important to note that the formation of chemically reactive metabolites does not necessarily result in appreciable toxicity. Often a reactive metabolite is fully degraded by enzymatic reactions or by binding to non-essential cellular sites or is excreted so that no observable injury occurs. Glutathione plays a particularly critical role in protecting cells against soft electrophiles and it is often only after the cellular GSH is considerably depleted that covalent reactions with other tissue nucleophiles is apparent. Several examples of non-toxic reactive metabolites are provided below. Further, when vital sites are affected, efficient repair processes may prevent critical damage, e.g., by removing damaged bases from DNA before the defect is fixed by DNA replication. Even without specific repair of damaged macromolecules, with time, disabled enzymes and receptors are resynthesized leading to the recovery of cellular functions.

7.2 Significance of Adduct Formation.

Much of the toxicological interest of active metabolites lies in those that are more reactive and which therefore bind covalently to form adducts (bound residues) with tissue macromolecules. Reactive metabolites are varied in structure and chemistry though most are electrophilic. Multiple nucleophilic sites exist on biological macromolecules that can act as sites for alkylation. The nature of the reaction sites for any active metabolite are critical in determining the type and severity of the resulting toxicity, if any.

Pearson's hard-soft acid-base concept (ref. 204) has been used as one guide to the types of reactions that different activated metabolites may undergo (refs. 9, 11). In particular, nucleic acids, containing relatively hard nucleophilic nitrogen and oxygen bases but lacking thiol groups and other soft nucleophiles, are most likely to react with hard electrophilic metabolites such as carbenium or nitrenium ions. Proteins and GSH, on the other hand, contain soft nucleophilic thiol groups. Soft electrophiles react predominantly with these thiols and the alkylation of nucleic acids cannot be detected. It is, of course, impossible to prove that no alkylation of nucleic acids occurs below the detection level, and small amounts of reaction with nucleic acids are to be expected even where thiol reactions strongly predominate (ref. 87). Nevertheless, when the active metabolite is a soft alkylating agent that preferentially reacts with thiol groups, there may be little risk of genotoxicity to the organism. Examples of such compounds include the quinone imine metabolite of acetaminophen (ref. 205), 2,4-dichlorophenacyl chloride (a metabolite of the insecticide, chlorfenvinphos; ref. 206) and the S-oxide metabolite of the triazine herbicide, cyanatryn (ref. 207). These compounds do not react readily with DNA and do not show mutagenic activity in typical tests. For example, cyanatryn S-oxide reacts covalently with hemoglobin in the rat but does not change its functions, cause immunological responses, react with DNA *in vitro* or act as a mutagen. Its bound residues in rat blood therefore appear to be without toxicological significance (ref. 87). This is particularly so since this type of binding is limited to rodents and birds and does not occur with human hemoglobin (ref. 88).

The sites of adduct formation by some reactive metabolites are surprisingly specific and only a few of the numerous potential sites of attack in the cell are modified. Skipper and Tannenbaum (ref. 208) concluded that while small reactive metabolites such as ethylene oxide or methyl bromide behave as free solutes and react without notable site specificity, larger, more lipophilic electrophiles tend to be relatively specific in their reactions due to their hydrophobic bonding (and steric) properties. This implies that a specific binding and orientation step precedes covalent linkage. Reactions may then occur with unexpected residues such as the *in vivo* binding of a reactive metabolite from 4-aminobiphenyl with the single, poorly nucleophilic tryptophan residue in rat serum albumin (ref. 209). Another example is the specific reaction of the herbicide metabolite, simetryn S-oxide, with the β-125 cysteine thiol of rat hemoglobin rather than with the more reactive thiol of cysteine β-93 (ref. 88). Finally, the carbodiimide formed from diafenthiuron reacts covalently with a single protein in rat liver mitochondria, a subunit of the F_1F_0-ATPase (ref. 79). This structural specificity in adduct formation has clear implications for varying and, as yet, rather unpredictable toxicological outcomes depending on the compound and species.

As highly sensitive methods are developed and refined to detect adducts, it is important to ask at what level the detection of altered macromolecules such as DNA or proteins becomes toxicologically significant. Their presence may be useful to gauge past exposure through molecular dosimetry (refs. 150, 208) but cannot be taken as a routine indicator of elevated risk since the significance of binding to different sites varies enormously. For example, all reactions with DNA are not equivalent toxicologically. Swenberg et al. (ref. 210) review studies showing that the alkylation of the ring nitrogens of DNA bases (N-7 of deoxyguanosine or N-3 of deoxyadenosine) show little correlation with tumor formation, but comparable alkylation of the exocyclic oxygen atoms (e.g. O-6 of deoxyguanosine) does correlate with mutagenesis and carcinogenesis.

The ability to correlate covalent binding to a specific toxic end-point is therefore variable. In some cases total binding reasonably correlates with observed toxicity. One such example concerns the liver carcinogen, aflatoxin B1 (AFB1), which is converted by microsomal monooxygenases to a reactive 8,9-epoxide as the ultimate carcinogen. There are distinct

species differences in sensitivity to this mycotoxin with the rat highly sensitive, the mouse relatively insensitive, and the hamster intermediate between the two. This rank order is reflected in several studies of total DNA adducts in liver slices and primary hepatocytes from these species (Table 2). The binding of AFB1 to DNA is much greater in rat tissue than in that from the mice. This is also true when AFB1 is administered *in vivo*. The hamster again is intermediate between rat and mouse. Additionally, male rats produce higher levels of DNA adducts than females in hepatocyte (but not liver slice) studies, which is in keeping with their higher hepatic monooxygenase activity. AFB1 is considered to be a carcinogenic risk to humans. Judging solely by the binding to hepatocytes, humans should be susceptible to AFB1 carcinogenicity with a sensitivity lying between that of the male rat and the mouse (ref. 211).

TABLE 2. Covalent binding to DNA in liver preparations from several species after exposure to aflatoxin B_1.

		Covalent Binding		
			Hepatocytes[a]	
	Carcinogenicity	Liver Slices[b]	I	II
Rat (M)	+ + + +	31.7	120[c]	203
Rat (F)	+ +	35.7	___	38
Hamster	+ +	11.3	___	___
Mouse	+	1.3	4.1	1.4
Human	?	3.4	20	49
		(0.7-8.5,n=6)	(13,27)	(25-69,n=3)

[a]Picomoles aflatoxin/mg DNA. Ref. 212(I), ref. 213(II)
[b]Nanograms aflatoxin/mg DNA. Ref. 211
[c]Sex unknown

Similarly, Bentley et al. (ref. 214) report higher levels of hepatic DNA adduct formation from p-chloro-o-toluidine (a metabolite of the carcinogenic pesticide, chlordimeform) in the mouse than the rat which corresponds to their relative sensitivity to the carcinogenicity of this compound. However, measurements of total protein or DNA binding generally include adducts at several or many sites that are of differing significance for the functions and survival of the cell. It is therefore not surprising that such gross measures may be inaccurate predictors of risk. For example, although variations in tissue binding with ^{35}S-labeled ANTU generally were correlated with lung pathology, pretreatment with ipomeanol completely protected rats from a typically lethal dose of ANTU but only reduced covalent binding of ^{35}S-ANTU to lung tissues by 15% (ref. 70).

With acetaminophen, although there is a general correlation of covalent binding to liver toxicity, treatments with some thiol agents and flavones prevent hepatotoxicity without a major reduction in acetaminophen binding (ref. 12). In a recent study, Roberts et al. (ref. 215) showed that 3'-hydroxyacetanilide, a non-hepatotoxic analog of acetaminophen, produced levels of cytochrome P-450-dependent covalent binding in the liver fully as large as those produced by acetaminophen. Clearly in this case covalent binding alone cannot be used to predict cytotoxic potential. Acetaminophen binds quite selectively to just a few proteins in mammalian liver. On the basis of several lines of evidence it has been concluded that covalent binding to a 55-58 kDa liver cytosol protein is closely correlated with hepatotoxicity (ref. 216). Sequencing of this protein indicates that it is homologous to a 56 kDa selenium binding protein (refs. 217, 218). How arylation of this protein leads to liver necrosis remains to be established.

It is well established that organophosphorus cholinesterase inhibitors phosphorylate many sites in vertebrate liver and serum which do not appear to contribute to the toxic effects of these compounds and which actually act as protective sinks for their removal (refs. 219, 220). Pretreatment with compounds that block these non-specific binding sites may increase the acute toxicity of organophosphates several-fold (ref. 219).

8. ACTIVE METABOLITES AND THE ASSESSMENT OF RISK

Many factors affect the rates of production and destruction of active metabolites and thus the duration and severity of any toxicological outcomes. These include the species, strain, age, and sex of the animal, its hormonal and nutritional status, the size and timing of exposures, and interactions with other chemicals in the animal's environment (ref. 221). Individual variations in sensitivity may be considerable for genetic and environmental reasons, particularly in human populations. A full discussion of these issues is beyond the scope of this paper. However, some illustrative examples are presented below to indicate how such variables may impact the ability to assess and manage risk in situations where active metabolites are responsible for the toxic end-point under consideration.

8.1 Size of Dose -- Pharmacokinetic Considerations.

A cornerstone of risk assessment is the definition of a dose-response relationship for the effect of concern. It is a requirement in some widely-used protocols for long-term animal carcinogenicity studies that the maximum tolerated dose (MTD) be used as the upper dose level. The MTD has been variously defined e.g. as the maximum dose that an animal can tolerate for a major portion of its lifetime without significant decrease in survival from effects other than carcinogenicity, without more than a 10% decrease in weight gain compared to controls, and without otherwise compromising the use of the study results in risk assessment (ref. 222). This dose is frequently several (three to five) orders of magnitude greater than the probable exposure of the general human population. Since active metabolites are often the cause of any observed toxicity, non-linearities in their production and destruction with applied dose make linear extrapolations potentially very inaccurate over such a wide range of exposures. In particular, the pharmacokinetics of active metabolites may generate effective "thresholds" for genotoxic (or other) endpoints that are not accommodated in current risk assessment practices based on non-threshold, linear extrapolation assumptions.

The simplest relationship between changes in the administered dose (exposure) and the level of active metabolite in the target organ is a linear (first-order) one (Fig. 22, curve 1). A linear relationship between dose and active metabolite levels (e.g. as indicated by adduct formation) may occur over several orders of magnitude. For example, adducts from cyanatryn (presumably formed via the sulfoxide) increased linearly over a dose range of 0.25 to 250 mg/kg (ref. 87) and Felton et al. (ref. 223) report linearity of DNA adduct formation over the dose range of 0.5 μg/kg to 100mg/kg with a naturally occurring heterocyclic amine that requires metabolic activation.

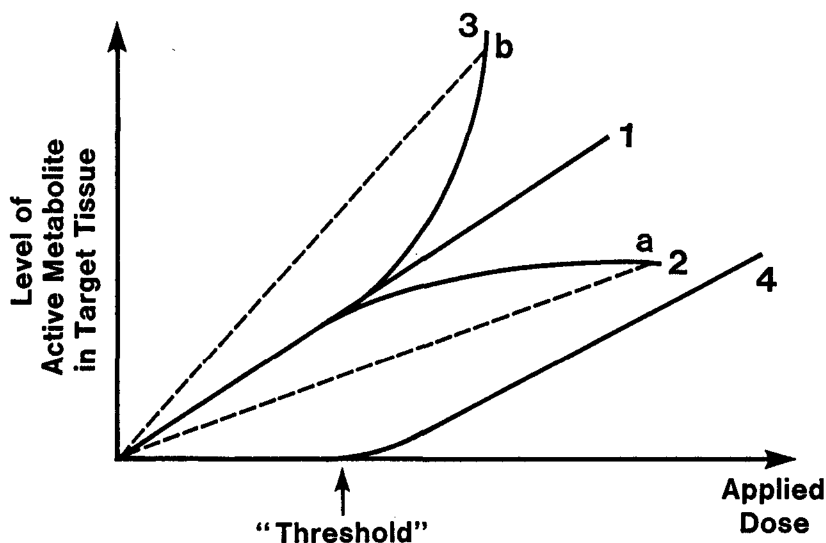


Fig. 22. Schematic representation of several possible relations between the applied dose of a protoxicant and the effective dose of the active metabolite at the target site. Dotted lines represent linear non-threshold extrapolations from points a and b.

However, it is obvious that as the dose of a protoxicant increases, many possibilities exist for non-linearities to arise in the delivery of the active product to the site of action. After a protoxicant is activated metabolically (and possibly transported to another tissue), it may be degraded either enzymatically or by reactions at non-essential sites that do not lead to toxicity, or it may react with an essential site with possible toxic outcomes (Fig. 2). Failure of the concentration of an activated metabolite to increase linearly with dose could arise if the activating enzyme(s) becomes saturated, if a co-substrate (e.g. GSH) of the activating enzyme is depleted, if product inhibition of the activating enzyme occurs, or because of selective induction of an enzyme that detoxifies the active product. In each case, as the administered dose rises, the concentration of the activated metabolite at the target organ will fail to keep pace and eventually no further increase will be seen despite further elevations in the dose (apparent zero order kinetics; Fig. 22, curve 2). Linear extrapolation from a high dose (point a) to low doses (dashed line) will then tend to underestimate the level of active metabolite at the target at lower doses and thus the likely extent of injury.

For example, some compounds that are converted to active metabolites by cytochrome P-450 are suicide substrates in that the enzyme product inhibits the enzyme that produces it. Examples include reactive sulfur from the oxidative desulfuration of phosphorothionates and carbon disulfide (ref. 70), activation products from carbon tetrachloride (ref. 12), and a carbene metabolite of the insecticide synergist, piperonyl butoxide (ref. 229). In contrast to the situation where systems that degrade the active metabolite are less efficient with dose, here the tendency is for activation efficiency to decrease with increasing dose, potentially leading to curves of type 2. In fact, such behavior was observed in mice with the phosphorothionate insecticide, fenitrothion, where the percentage of the administered dose undergoing thiono oxidation decreased considerably as the dose was increased (ref. 230).

A similar effect is seen when the activation mechanism is saturated by higher doses of the parent causing a transition from first-order to zero-order kinetics. Examples of this include the conversion of the plant growth regulator, ethylene, to ethylene oxide (ref. 231), the comparable oxidation of vinyl chloride (ref. 232), and activation of a tobacco smoke constituent, 4-(N-methyl-N-nitrosoamino)-1-(3-pyridyl)-1-butanone (ref. 233.

On the other hand, if any of the same events tend to decrease the rate of destruction of the parent compound or the active metabolite by enzymatic or tissue binding reactions, it will lead to the opposite effect (Fig. 22, curve 3), in which the level of active metabolite will rise more rapidly than expected as the dose increases (sometimes termed a "hockey-stick" curve). In this case, high (point b) to low dose linear extrapolation (dashed line) overestimates the active metabolite level that will be present at lower doses and the toxicity seen at high doses may have little relevance for environmental exposure levels. In a more extreme, but not unrealistic, situation (curve 4), this behavior results in an effective threshold below which no detectable amount of the active metabolite is present and thus no toxic response might be expected. The unequivocal experimental demonstration of such thresholds is technically impossible because all methods of detection for the active metabolite or its products have limits of sensitivity. However, several examples of non-linear relationships approximating that of curve 4 are known and it is likely to be a relatively common situation in active metabolite-generated toxicity.

One example is provided by the hepatic release of paraoxon, the activation product of parathion. At low doses, paraoxon is destroyed by phosphorylation of macromolecules within the liver parenchymal cells and none can be detected leaving the liver. Only when these reaction sites are fully phosphorylated does the activated ester enter the systemic circulation (ref. 220). Release of the hepatically generated reactive metabolite to the other tissues thus has an effective break-though point or threshold.

Apparent thresholds for active metabolite binding in the liver are seen with acetaminophen (ref. 224) and bromobenzene (ref. 225). In each case these compounds are oxidized in the liver to potentially reactive alkylating agents (a quinone imine and an epoxide, respectively). However, these reactive metabolites are efficiently removed by reaction with hepatic GSH. Only when this GSH is depleted by 70% or more is covalent tissue binding and liver necrosis detectable. This non-linear relation of adduct formation to dose with an effective threshold has

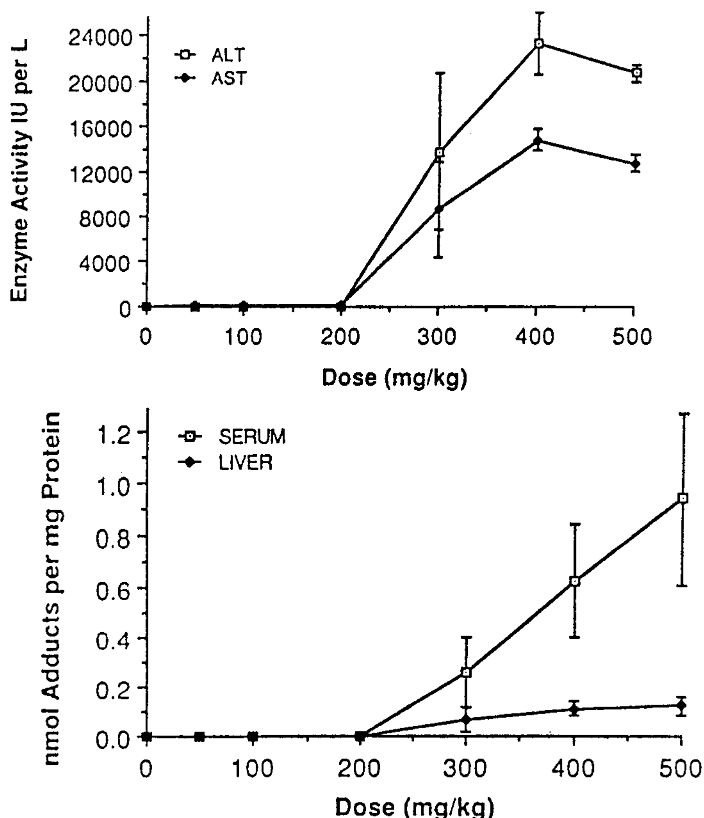


Fig. 23. Non-linear relationship between hepatotoxicity and applied dose in male mice treated intraperitoneally with acetaminophen.
A. Occurrence of liver enzymes in serum four hours after treatment as an indicator of hepatotoxicity. ALT = alanine aminotransferase and AST = aspartate aminotransferase.
B. Adduct formation in liver and serum proteins in the same mice. The level of arylated protein in the liver was determined by specific immunoassay for the cysteinyl adduct. From ref. 226, with permission.

been clearly illustrated by Pumford et al. using an immunoassay for the cysteinyl-S-acetaminophen conjugate. The dose-response relationship for conjugate formation and indices of hepatotoxicity are in full agreement (Fig. 23; ref. 226).

In a further example, at low doses, o-phenylphenol is eliminated by conjugation with glucuronic acid or sulfate. However, at high doses, glucuronidation and sulfation cannot increase proportionally with dose. As phenylphenol accumulates, oxidation to form a reactive quinone (Fig. 13) is encouraged resulting in bladder hyperplasia and tumors. This is clear from the fact that at oral doses in rats of 5 or 50 mg/kg of the sodium salt, OPP-Na, no detectable amount of o-phenylphenol is converted to metabolite III as an indicator of the ring hydroxylation that leads to quinone formation. At 500 mg/kg approximately 25% of the dose is converted to this metabolite (ref. 227).

Also, tetrachloroethylene is metabolized by two competing routes, epoxidation and GSH conjugation. Epoxidation appears not to result in genotoxic effects. Much of the reactive oxidation product is trapped as trichloroacetyl adducts with phospholipids. However, GSH conjugates are converted to a carcinogenic metabolite in the kidney (ref. 58). At low doses, epoxidation by far predominates as the route of metabolism and the products of GSH conjugation only become apparent at high doses that do not represent likely environmental exposures (ref. 228). Risk assessments for nephrocarcinogenicity based on responses at high doses therefore are likely to overestimate risks at low doses.

If either the parent compound or a metabolite can act as an inducer of metabolic enzymes such as P-450 monooxygenases, GSH transferases or glucuronyl transferases, rates of activation, detoxication or both could change considerably after sufficient time (a few hours) has elapsed to allow new enzyme synthesis. The potential significance of such induction on the dose-response curves of protoxicants is clear, but specific examples do not seem to be well established.

8.2 Significance of Non-Linear Activation Relationships for Risk Assessment.

The significance for risk assessment of non-linear dose-related events due to metabolic activation has been discussed by a number of authors (210, 232, 234-238). It is difficult to disagree with the general conclusion that for optimum risk assessment, responses should be related to the level of active metabolite at the site of action (the "molecular dose") rather than the applied dose of protoxicant given to the organism. Such non-linearities can be incorporated into pharmacokinetic or more detailed physiologically based models describing the intoxication process (refs. 236, 238, 239). Considerable time and effort must be expended to develop the necessary information for this type of analysis and currently it is not a routine procedure, but it remains an essential goal for the development of more insightful and accurate risk assessments based on biological realities rather than mathematical abstractions.

8.3 Variations between Species.

The second area of concern regarding the accuracy of risk assessments based on animal studies is that of interspecific differences, particularly between rodents, as the common test species, and humans. This has been covered in detail in a monograph by Miyamoto et al. (ref. 31). It is common in current risk assessment practices to include a 10-fold uncertainty (safety) factor for the extrapolation of toxicity data from animals to man and a further 10-fold factor to allow for the greater individual variability in human populations (ref. 236). Though founded on toxicological experience, these uncertainty factors are arbitrary and not based on experimental data derived from the specific compound and species under study. A more detailed understanding of comparative toxicology would reveal that safety margin of this magnitude are unnecessary in many cases yet may be inadequate in others.

Few metabolic reactions are unique to one species although some reactions such as the glutamine conjugation of arylacetic acids, the N-glucuronidation of sulphadimethoxide, the aromatization of quinic acid, and the O-methylation of 4-hydroxy-3,5-diiodobenzoic acid seem to be restricted to humans and non-human primates (ref. 31). On the other hand, as already mentioned, some species are deficient in certain reactions that can be important in activation (Table 1).

More generally, rather than finding qualitative differences in the metabolism of a compound between species, quantitative variations exist in the rates and relative emphasis on common pathways of metabolism. This may still lead to considerable differences in the rates of generation and destruction of active metabolites. To illustrate this point, Fig. 24 shows the relative activities of liver P-450 monooxygenases from a range of mammals. They represent ranges of activity determined with several substrates (ref. 31). However, it must be again emphasized that the cytochrome P-450s consist of a complex mixture of isozymes in most mammalian tissues and broad generalizations about relative activities in different species by no means indicate that every reaction catalysed by P-450 will follow the same pattern.

The trend for larger species (including humans) to have lower monooxygenase activity per unit body weight is clear and this is mirrored in the slower clearance in humans of xenobiotics metabolized by liver monooxygenases (refs. 31, 235). This conclusion has been supported by studies comparing aminopyrine demethylation across a range of mammals (ref. 242). A negative linear relation between body weight and the rate of demethylation *in vivo* was observed with a 100-fold difference in rate between mice and rabbits. This variation presents a challenge in risk extrapolations to humans since most toxicity tests are performed with small rodents. The fact that monooxygenases may be involved both in activation and detoxication reactions with a single substrate adds a further complication to this scaling correction.

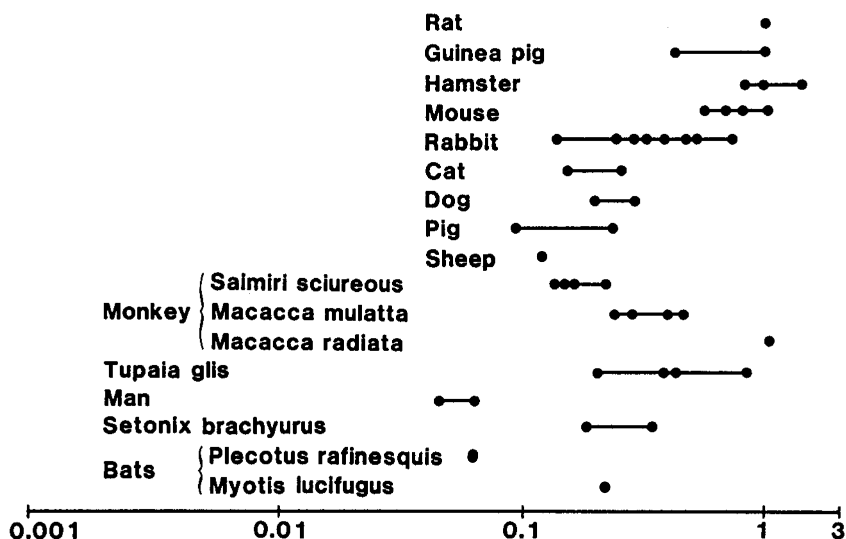


Fig. 24. Comparative activities of P-450-catalysed monooxygenase reactions in mammalian species based on observations with several substrates. The scale is relative to activity in the male rat = 1.0 and is adjusted for different liver:body weight ratios in the different species. From ref. 31, with permission.

The data in Table 3 suggest that glutathione transferase activity is also lower in the human liver than in many other species, but this lower activity is not generalizable to all xenobiotic-converting enzyme systems e.g. human liver epoxide hydrolase activity is considerably higher than that in many other vertebrates and glucuronyl transferase activity is comparable to that in rodents.

TABLE 3. Comparative activities of several enzymes that activate xenobiotics in the livers of mammals. Based on data from ref. 31.

Species	GSH Transferase[a]	Epoxide Hydrolase[b]	Glucuronyl Transferase[c]
Mouse	149	1.9	0.44
Rat	111	5.2	0.33
Rabbit	31	4.2	0.78
Pig	11	6.4	----
Dog	19	9.7	----
Guinea Pig	287	12.0	2.50
Baboon	7	31.3	1.10[d]
Hamster	107	12.6	----
Human	25	12.7	0.66

[a]Substrate; styrene oxide (nmol min^{-1} mg $protein^{-1}$). Cytosolic enzymes.
[b]Substrate; styrene oxide (nmol min^{-1} mg $protein^{-1}$). Microsomal enzymes.
[c]Mean rate for several substrates; relative activity adjusted for liver/body weight ratio. Microsomal enzymes.
[d]Rhesus monkey

Again it is important to be aware that the rankings of species by activity may vary greatly with small changes in the substrate or tissue used and that broad generalizations are dangerous, even though large species variations in rate do exist and have toxicological relevance. One example of unpredictable species variation is the A-esterase activities of liver microsomes when assayed with the methyl and ethyl homologs of paraoxon. The mouse and rabbit preferentially hydrolyse the ethyl analog, the horse has a very high relatively specific activity against the methyl analog and the chicken is entirely specific for this analog (ref. 243). Several other interspecies comparisons in the enzymology of xenobiotic metabolism are presented by Miyamoto et al. (ref. 31).

Species variations in activation routes and rates clearly have important toxicological consequences. To cite one example, carbon tetrachloride has been shown to be a liver carcinogen in numerous animal studies. Hamsters are the most sensitive species studied, followed by mice and then rats. This correlates with relative activity in carbon tetrachloride activation by monooxygenases. Hamsters tend to have a particularly high hepatic P-450 monooxygenase activity (Fig. 24) and show higher levels of covalent binding to liver nuclear proteins than the other species after exposure to [^{14}C]CCl_4 either *in vitro* in an NADPH-dependent reaction, or *in vivo* (Table 4). Interestingly, there is no difference in binding of CCl_4 to liver DNA between these species, suggesting that the alkylation of nuclear proteins is the critical event in hepatotoxicity (ref. 244).

TABLE 4. Comparative covalent binding of carbon tetrachloride to liver macromolecules in three mammalian species (data from ref. 243).

	Covalent Binding To			
	Nuclear Proteins			DNA
	In vitro		*In vivo*	*In vivo*
Animal	-NADPH	+NADPH		
Syrian Hamster	193	625	7.5	2.3
C3H Mouse	122	223	6.1	2.3
Sprague Dawley Rat	48	168	1.8	2.2

[a]pmole CCl_4/mg liver nuclear protein
[b]pmol CCl_4/mg liver DNA

Another example of species differences in activation leading to decisive differences in risk is found in tetrachlororethylene which causes kidney cancer in male rats but not in mice. One probable cause lies in the generation of glutathione conjugates subject to further β-lyase action in the kidney, as previously described. It is notable that rats form glutathione conjugates of tetrachloroethylene much more readily than mice. They also have a higher kidney β-lyase activity. It is reassuring that human liver does not form such conjugates, suggesting that we are subject to little risk of this type of toxicity (ref. 239).

Clearly, risk assessment protocols that inflexibly extrapolate human risk using responses from the most sensitive test species will not produce realistic results in a case such as these. Recognition of differences in the metabolism of protoxicants in test animals and humans, preferably combined with comparative pharmacokinetic analysis, is critical for accurate risk extrapolation.

8.4 Gender-Based Variations in Metabolic Capabilities.

Differences between the sexes in metabolism of xenobiotics often are relatively small, but in some cases may be significant. Several sex-specific forms of P-450 have been identified and these are under regulatory control of the sex hormones (ref. 22). While male rats have higher levels of some forms of cytochrome P-450 than females, this situation is reversed for an important subgroup of P-450 forms (P-448 or P450I). These forms are particularly important in activating acetaminophen and this may explain while female rats are more sensitive to this compound than males (ref. 24).

In another example of sex-related differences, the sulfation of N-OHAAF (phenol sulfotransferase activity) is deficient in female compared to male rats, and this correlates well with the greater sensitivity of males to N-OHAAF-induced hepatotoxicity. Other substrates are sulfated equally well by the two sexes (ref. 244). Further, female rats have a higher liver cytosolic sulfotransferase activity (hydroxysteroid sulfotransferase) than males when phenolic metabolites of some polycyclic aromatic hydrocarbons are used as the substrate. Sulfation in this case also forms highly active carcinogens (ref. 246). This illustrates the risks in generalizing about levels of activity in whole reaction classes when multiple enzyme forms are involved. Such sex-related differences in metabolism are often less marked in other vertebrates including humans, but this varies with the compound under study. Gender-related differences in xenobiotic metabolism in humans have recently been reviewed (ref. 247).

8.5 Individual Variations.

Differences between individuals in xenobiotic metabolism are considerable in human populations due to a combination of genetic and environmental factors. A study of the rates of N-hydroxylation of arylamines in 22 individuals illustrates this range. With 4-aminobiphenyl, there was a 110-fold variation in rate between the least and most active liver microsomes while the difference was 9-fold when 4,4'-methylenebis(2-chloroaniline) was the substrate. This implies considerable individual variation in risk after exposure to potentially carcinogenic arylamines (ref. 248). A subsequent examination of several individual forms of P-450 and epoxide hydrolase in human liver samples again showed a very broad range of activities among individuals. Liver cirrhosis decreased most P-450 isozyme levels but P-450 IIC was elevated in cirrhotic livers and those from cancer patients (ref. 249). The data for AFB1 in Table 2 also illustrate differences between individuals with varying capabilities to form DNA adducts. A 12-fold range was seen in liver slices, even though only six individuals were in the sample. Such variation is typical of other xenobiotic-metabolizing enzymes, e.g., phenol sulfotransferase activity varies widely between individuals in human populations with over 15-fold difference in activity in the enzyme from platelet cytosol (ref. 53). Metabolic rates in inbred, closely age-matched experimental animals do not show comparable variability. An allowance for this greater variability must obviously must be made in human risk assessments. The common use of a rule-of-thumb allowance of a 10-fold factor has already been mentioned.

Part of the wide metabolic variability between individuals in human populations results from the existence of genetic polymorphisms in metabolic enzymes, e.g., genotypes are well known for fast and slow acetylation of aromatic amines and for fast and slow debrisoquine hydroxylation. These polymorphisms are differentially present in various human racial and geographic groupings. This is discussed in detail in refs. 31 and 250. Several polymorphisms in cytochrome P-450 have also been identified in rats and other vertebrates (ref. 22). This is a topic of considerable current interest and many more polymorphisms will eventually be defined using the tools of molecular genetics. From this may arise a much more personalized ability to predict and minimize risk from exposure to specific types of toxicants.

Another factor that causes variations in activative reactions is age. This affects metabolic capabilities, with neonate and aged animals having lower activities in some reactions involving glucuronidation and of many, but not all, forms of cytochrome P-450 (refs. 24, 31). The implications of this for the toxicity of protoxicants are likely to be complex and dependent on specific combinations of age, species and compound.

8.6 Environmental Influences on Activation Reactions.

Items falling under this category are so many and diverse as to defy more than coverage in outline. They include such factors as diet, exposure to varied environmental contaminants, medications, alcohol, disease states and physical stress. Very few studies address agrochemicals in this context. Most focus on the effect of these variables on drug metabolism and relatively few specifically address activation reactions, but a handful of examples are provided to at least illustrate the effect that environmental changes may have on the metabolic systems that are known to carry out activations. It is clear that only by developing an understanding of the effects of environmental variables on specific isozymes of xenobiotic-metabolizing enzymes can a proper interpretation be made of their implications for altered toxicity.

Many studies have been conducted to evaluate the effects of dietary variables on cytochrome P-450 isoenzymes and drug metabolism. Monooxygenase activities generally decline in animals fed a diet rich in protein. Fasting, pregnancy and diabetes all lead to increased expression of specific monooxygenase enzymes in the rat, although the toxicological significance is not well established (ref. 22). In a specific example relating to diet, rats reared on a diet deficient in sulfur-containing amino acids showed an increased sensitivity to the hepatotoxicity of acetaminophen, probably because of the decrease in protective thiols that react with the quinonoid activation product (ref. 251).

Many xenobiotics present in the diet, including natural products, can induce liver enzymes, particularly specific forms of P-450-dependent monooxygenases. This has obvious implications for activation reactions, e.g., polycyclic aromatic hydrocarbons such as 3-methylcholanthrene increase the toxicity of acetaminophen to mice by enhancing the level of a specific isozyme of P-450 (P-450IA) which activates it (ref. 24, p. 42). The medicinal agent, phenobarbital, which induces P-450II types, does not increase this toxicity. Plant-derived flavanoids induce certain P-450 isozymes and increase the activation of compounds such as benzo[a]pyrene and aflatoxin B_1 (ref. 252) while plant phenolics inhibit this effect (ref. 253).

Alcoholics show a greater sensitivity to acetaminophen than the general population and may suffer hepatotoxicity at normal therapeutic doses (ref. 226). One reason may be that alcohol consumption in rats leads to the induction of cytochrome P-450IIE1. This P-450 isoform is particularly capable of activating acetaminophen. It also reduces carbon tetrachloride rapidly (ref. 254) which suggests another elevated risk factor in alcoholism. Smokers show a relatively specific increase in the activity of cytochromes P-450IA1 and P-450IA2 (ref. 22) which are capable of activating a broad range of xenobiotics with well established significance for the generation of genotoxic metabolites (ref. 24).

Other examples of the effect of environmental variables on xenobiotic metabolism are cited in refs. 9, 22, 24, 255 and 256. Dietary and nutritional influences are reviewed in refs. 257-259.

In conclusion, multiple factors based on genetics, age, environmental factors, and the route and intensity of the exposure affect the generation and destruction of active metabolites. This raises a dauntingly complex challenge of understanding and interpretation to the metabolism chemist and the toxicologist. Only through a better knowledge of the characteristics of metabolic systems and their reaction products in experimental species and humans can we hope to attain a high degree of predictability in risk assessment. The accurate understanding of the quantitative toxicological role of active metabolites is particularly demanding. However, if risk assessment is ever to move from the gross approximations and multiple, generally conservative assumptions of current approaches to a more accurate, biologically-based science, these complexities must be addressed. Even an approximation of biological reality is preferable to the "black box" methodologies and broad, often conservative approximations that connect the administered dose and the observed effect in most current approaches to risk assessment. However, it is unlikely that these demanding attempts to understand the biological basis of toxicity will be pursued at the level necessary for success unless regulatory agencies are prepared to reward the effort by adopting well-founded biologically-based approaches to risk assessment as they become established.

9. CONCLUSIONS

1. Although the biological effects of agrochemicals, such as pesticides and animal drugs, are often due to the direct actions of the parent compound, in many cases metabolites are primarily responsible for both intended and unintended toxicity.

2. The rates of production and destruction of these active metabolites may greatly influence the rate of elimination and the nature and severity of the toxicity of an agrochemical.

3. Known bioactivation processes can sometimes be exploited in the development of propesticides that require metabolic activation *in vivo*. This provides an opportunity for enhanced selectivity leading to safer pesticides with improved environmental properties.

4. Active metabolites are formed primarily by oxidative enzyme reactions, and particularly by monooxygenases containing cytochrome P-450. However, hydrolysis, reduction, and conjugation with such entities as glutathione or sulfate may be important in specific cases. Multiple enzyme-catalysed steps are involved in the generation of some important activated metabolites.

5. The intrinsic reactivity and biological potency of an active metabolite are not necessarily connected. Highly reactive metabolites are not always highly toxic. Bound residues that

result from the interaction of activated metabolites with tissue macromolecules may act as markers of exposure and bioactivation, but are not necessarily of toxicological significance. This point is particularly important as increasingly sensitive methods are developed to detect and quantify the adducts produced by reactive intermediates. The interpretation of the significance of tissue adducts is still difficult and controversial.

6. The very high intrinsic reactivity of some activated metabolites may make them difficult to detect and quantify directly. In some cases their formation can only be inferred from indirect evidence.

7. The critical active metabolite may represent only a small fraction of the total metabolites of the parent compound and will correspondingly be difficult to detect and monitor. For purposes of risk assessment and management it is essential to discover and define these critical, if quantitatively minor, pathways.

8. In many cases active metabolites are discovered only after the exposure of organisms to new chemicals has occurred, but, increasingly, such reactions can be predicted on the basis of accumulated knowledge of the metabolic fate of xenobiotics, the reactions of their metabolites, and simple *in vitro* tests.

9. Active metabolites are of central importance in understanding both the acute and chronic toxicity of agrochemicals in target and non-target organisms, and in the proper extrapolation of toxicity between species, including humans. Early identification of the existence of active metabolites is important in planning and interpreting long term toxicity studies.

10. Risk assessment protocols that adopt a rigid mathematical approach for dose-response analysis without addressing the biological events underlying the response of concern may misjudge the potential for toxicity outside the range of the observed data. This is particularly true when activated metabolites are involved in producing the toxic response. Linear extrapolation from high doses to low doses is unlikely to be correct in many cases and effective thresholds may occur in dose-response relationships below which no toxicity is likely to occur.

11. An understanding of the nature, modes of toxic action, and rates of production and destruction of active metabolites on a comparative basis between experimental animals and humans is another essential prerequisite for scientifically-based and biologically-realistic risk assessment. Methods to study xenobiotic metabolism in humans are increasingly available e.g. through the use of stable isotopes *in vivo*, human metabolic enzymes from cloned genes, transgenic human cell lines and assays for tissue adducts. This knowledge is also essential in understanding potential interactions of chemicals in multiple exposures, and the effects of environmental variables on responses to agrochemicals.

10. RECOMMENDATIONS

1. Appropriate consideration should be given by agrochemical companies and agencies involved in planning toxicity studies and regulating agrochemicals to the potential role of active metabolites in generating toxic end-points of concern. This is particularly important if there are relevant indicators of metabolic activation such as bound residues.

2. Improved methods are needed for the identification and quantitation of active metabolites to assist in defining the shapes of dose-response curves at environmentally-realistic exposure levels. The greatest need is for methods to detect and study metabolites that are highly reactive and can only be investigated indirectly.

3. Bound residues (adducts) are not necessarily an indicator of toxicity, but they do indicate the need for additional studies to clarify their origins and significance. Greater effort is needed to determine the toxicological significance of bound residues derived from active metabolites in animal tissues.

4. In order to assess the significance of active metabolites observed in animal studies, high priority should be given to improving knowledge of human capabilities to produce and destroy active metabolites. This necessarily involves studies on human subjects and tissues with appropriate consideration of ethical requirements. Emphasis should be placed on developing methodologies that allow human studies to be conducted safely.

5. To achieve more accurate and rational risk assessment and optimum risk management, greater effort is needed to understand the occurrence and significance of active metabolites of agrochemicals especially at realistic environmental levels of exposure. Risk assessment should take into account the biological basis of toxicity, including the dosage of active metabolites in the target organ and comparative metabolism in animals and humans. The increased use of comparative pharmacokinetic analysis in risk assessment is necessary. This is desirable in order to predict potential toxicity over a variety of exposure levels and routes, and a broad range of environmental conditions, and to extrapolate results between species.

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