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TOXICOLOGY OF THIONO-SULFUR COMPOUNDS

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INTRODUCTION

A number of thiono-sulfur (C=S or P=S) containing compounds which find use as pesticides or drugs and in industrial processes exhibit toxic properties in mammals. These effects include bone marrow depression, liver damage, lung damage, induction of neoplasia, and inhibition of various enzymes, including the cytochrome P-450 containing monooxygenases. There is considerable evidence that metabolism of the thiono-sulfur group is a prerequisite for manifestation of most of the toxic effects of thiono-sulfur compounds. Therefore the emphasis in this review will be placed on recent developments concerning the pathways by which these compounds may be converted to toxic metabolites and how these metabolites may interact with biological systems. After a brief review of the different adverse effects associated with exposure of experimental animals and humans to thiono-sulfur compounds, the evidence supporting a critical role for metabolic activation in mediating the toxicity of this class of compounds will be summarized. The final section will deal with recent studies of the metabolism and biochemical mechanisms of toxicity of three representative thiono-sulfur compounds: carbon disulfide, parathion, and thioacetamide.

ADVERSE EFFECTS OF THIONO-SULFUR COMPOUNDS

Bone Marrow Depression

A common property of nearly all thiono-sulfur-containing compounds, including the naturally occurring compound goitrin (1), is that they inhibit the iodination of tyrosine in the thyroid gland and, consequently, prevent thyroxine synthesis. This is the basis for use of thiono-sulfur-containing

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compounds in the treatment of thyrotoxicosis. Bone marrow depression has been a common side effect of the use of thiono-sulfur containing compounds in the treatment of thyrotoxicosis in man. Those thiono-sulfur-containing compounds most commonly in use currently are propylthiouracil and methimazole. Reports of bone marrow depression with these compounds are somewhat less frequent than was the case with thiouracil, methylthiouracil, and thiobarbital, compounds which are no longer used because of their high toxicity. However, reports of bone marrow depression with propylthiouracil and methimazole do appear in the literature. For example, Wiberg & Nuttall (2) have reported that 8 of 25 patients treated with methimazole had severe toxic reactions, including two cases of agranulocytosis syndrome. Amrhein et al (3) have reported that of 38 patients aged 2 to 18 years treated for hyperthyroidism with propylthiouracil, 10 (26%) had one or more episodes of leukopenia and 7 (18%) were granulocytopenic.

Liver Damage

A number of thiono-sulfur compounds, including thioacetamide (4), carbon disulfide (5–7), methimazole (7), propylthiouracil (7), diethylphenyl phosphorothionate (8), and thiobenzamide (9) have been shown to cause centrilobular hepatic damage upon administration of a single dose to laboratory animals. The liver damage caused by carbon disulfide and diethylphenyl phosphorothionate is observed only in rats pretreated with phenobarbital, and has been described as periacinar hydropic degeneration (8). Thioacetamide, on the other hand, produces frank necrosis, even in rats which have not been pretreated with phenobarbital, although such pretreatment does enhance toxicity (10). There are several reports of liver damage in man following administration of thiono-sulfur compounds for therapeutic purposes. Such compounds include thiobarbital (11), disulfiram (12), methimazole (13), propylthiouracil (14), methylthiouracil (15), and thiouracil (16). Disulfiram (antabuse) is currently used in the treatment of alcoholic patients, and the remainder of the above drugs have been or are currently being used in the treatment of thyrotoxicosis. It is not clear from the reports whether the hepatic injury in humans was a direct result of the action of the compounds or a hypersensitivity reaction.

The acute administration of α-naphthylisothiocyanate (ANIT) to several animal species results in cessation of bile flow (cholestasis) accompanied by increases in plasma levels of cholesterol, bile acids, and bilirubin. Upon chronic administration bile duct hyperplasia and biliary cirrhosis are observed. The literature dealing with the acute cholestatic response has been reviewed by Plaa & Priestley (17). They conclude that the acute action of ANIT is largely due to an effect on hepatocyte function.

Lung Damage

Some thiono-sulfur compounds produce lung edema in mammals. Most notable of these compounds are thiourea (18) and the rodenticide α-naphthylthiourea (ANTU) (19). ANTU produces a fibrin-rich pulmonary edema with pleural effusion in rats (19, 20) and dogs (21). Examination of the pathology in rats suggests that the pulmonary edema is due to the formation of reversible gaps in the endothelium of small pulmonary vessels as a result of damage to the pulmonary vascular endothelium (22). An additional study (23) revealed that as early as 2 hours following ANTU administration a blebbing and scalloping of endothelial cells and interstitial edema can be seen. By 6 hours epithelial damage is also apparent.

Cancer

A number of thiono-sulfur-containing compounds have been shown to produce cancer in experimental animals. These include thiourea, thioacetamide, ethylene thiourea, propylthiouracil, thiouracil, and methylthiouracil (24). Cancer of the thyroid, liver, kidney, vagina, pituitary, and zymbal glands has been reported. Adequate case reports or epidemiological studies for evaluating the carcinogenicity of the above compounds in humans appear to be lacking.

Inhibition of Cytochrome P-450 Dependent Monooxygenases

Representatives of the thiono-sulfur-containing compounds which have been shown to inhibit the cytochrome P-450 containing monooxygenases and to decrease the level of cytochrome P-450 detectable as its carbon monoxide complex when administered in vivo to experimental animals are carbon disulfide (5–7, 25), disulfiram (7, 26), methimazole (7), diethyldithiocarbamate (7), thiourea (7), ethylene thiourea (7), thiouracil (7), methylthiouracil (7), propylthiouracil (7), α-naphthylthiourea (27), thioacetamide (28), various phosphorothionate insecticides (8, 29), α-naphthylisothiocyanate (30), and ANTU (31). With the exception of thioacetamide, the above compounds also cause inhibition of cytochrome P-450 when incubated with hepatic microsomes in vitro in the presence but not the absence of NADPH. This suggests that the inhibition reflects a primary effect of metabolism of the compound, rather than a secondary effect of the liver damage caused by many of the compounds.

Other Adverse Effects

Carbon disulfide presents a rather special case as concerns thiono-sulfur-containing compounds. It has been used industrially over the last 100 years as a solvent in various industrial processes, in the cold vulcanization of rubber, and in the manufacture of viscose rayon. Many toxic effects have

been attributed to exposure to this compound. In addition to those we have already cited (enzyme inhibition and liver damage), CS_2 has been reported to cause neurological effects, visual defects, gastrointestinal symptoms, nephrosclerosis, clinical hypercorticism, anemia (32), an increase in coronary heart disease in man (33, 34), and testicular atrophy in rats (35).

ROLE OF METABOLISM IN TOXICITY OF THIONO-SULFUR COMPOUNDS

During the past decade it has become increasingly evident that metabolism of the thiono-sulfur group plays a fundamental role in eliciting the toxic effects of thiono-sulfur compounds. The experimental support for this conclusion is based on four different lines of investigation, including: (*a*) the effects of inhibitors and inducers of metabolism on toxicity; (*b*) the comparative toxicity of analogs (especially the oxygen analogs); (*c*) direct toxicity testing of intermediate metabolites of sufficient stability to be isolated; and (*d*) covalent binding of metabolites of thiono-sulfur compounds in vitro and in vivo. These various approaches will now be discussed individually as they have been applied to representative thiono-sulfur compounds.

The Effects of Inhibitors and Inducers of Metabolism on Toxicity

Phenobarbital pretreatment has been shown to enhance the desulfuration in vivo and/or in vitro of representatives of most of the major classes of thiono-sulfur compounds including parathion (36, 37), carbon disulfide (6, 25, 38), thioacetamide (39), and α-naphthylisothiocyanate (ANIT) (40). Consistent with these findings, phenobarbital has been found to enhance the liver damage produced by diethylphenyl phosphorothionate (a parathion analog) (8), and by carbon disulfide (5, 7, 41, 42), thioacetamide (10), and ANIT (40). However, in this context it should be pointed out that the effect of phenobarbital on carbon disulfide toxicity might not be mediated through its transformation to a more toxic metabolite, but may result from an increased susceptibility to the action of CS_2 itself (42, 43).

The effects of inhibitors of metabolism on the organ damage caused by carbon disulfide, thioacetamide, ANIT, and α-naphthylthiourea (ANTU) are also consistent with a requirement for metabolic activation of the compounds. The inhibitor SKF-525A has been shown to give some protection against the toxicity of all four compounds (5, 10, 40, 41, 44). Piperonyl butoxide has been used to protect against the toxicity of ANIT (40) and ANTU (44), and cobaltous chloride against the toxicity of ANIT (40) and thioacetamide (10).

Consistent with the above results, pretreatment of rats with small nonlethal doses of ANTU was found to inhibit the in vitro and in vivo metabolism

of ANTU, and to protect against the lethality and lung damage of subsequent normally lethal doses (27).

Comparative Toxicity of Analogs of Thiono-Sulfur Compounds

Studies of mono- and disubstituted thioureas suggest a good correlation between acute toxicity and desulfuration in vivo. Thus the toxic monoarylthioureas (phenyl, *p*-chlorophenyl- and *p*-tolyl-) were desulfurized to a considerable extent (66–100%) upon in vivo administration to rabbits, whereas the relatively nontoxic diarylthioureas (diphenyl- and 4-hydroxydiphenyl-) were not (<25%). Similarly, *p*-hydroxyphenylthiourea, which was at least five
found to undergo only a slight degree of desulfuration (45).

The most compelling evidence that the thiono-sulfur group is required for toxicity is provided by a comparison of the potency of thiono-sulfur compounds with their corresponding oxygen analogs. In many cases these oxygen analogs are also the major metabolites found in vitro or in vivo. With the exception of the anticholinesterase activity of the phosphorothionate and phosphothionate insecticides, which is greatly enhanced upon conversion to the oxygen analogs, the toxicity of thiono-sulfur compounds is greatly reduced or abolished upon replacement of sulfur by oxygen. For example, the 24 hour LD_{50} for intraperitoneal administration of ANTU to male Sprague-Dawley rats was found to be 10 mg/kg, whereas the value for the oxygen analog ANU was > 800 mg/kg. Moreover, at this dose ANTU caused severe pulmonary edema, whereas none was observed after ANU administration (27). Similarly, replacement of the sulfur atom of ANIT with oxygen abolished the hepatic dysfunction, as judged by the absence of hyperbilirubinemia (17). The inhibitory activity towards cytochrome P-450 in vivo and/or in vitro of a number of thiono-sulfur compounds including thiourea, ethylene thiourea, thiouracil, methylthiouracil, propylthiouracil, phenythiourea, parathion, ANTU, and ANIT is abolished upon replacement of sulfur by oxygen (7). Finally, substitution of oxygen for sulfur abolishes the carcinogenicity of thiourea and thiouracil, and greatly decreases the potency of thioacetamide.

The low order of toxicity of oxygen analogs of thiono-sulfur compounds, on the one hand, and the good correlation between desulfuration in vivo and toxicity, on the other hand, are of interest for two reasons. First, they demonstrate that the toxicity of thiono-sulfur compounds is inherent in the thiono-sulfur group. Second, they strongly implicate reactive intermediates formed during the desulfuration process as being responsible for toxicity. In a later section, direct evidence for the production of such intermediates based on the observation of covalent binding will be discussed.

Direct Toxicity Testing of Intermediate Metabolites

Some thiono-sulfur compounds are metabolized to S-oxides of sufficient stability to be chemically synthesized and tested directly for toxicity. Thus it has been reported that rats exposed by inhalation to the S-oxide of the pulmonary toxin, thiourea, exhibit pathological changes similar to those observed with the parent compound and that the S-oxide is at least 10-fold more potent (46). Similarly, the S-oxide of thioacetamide has been shown to be a more potent hepatoxin than thioacetamide itself. Thus at equimolar doses thioacetamide S-oxide produces a more rapid onset and a greater severity of centrilobular necrosis than thioacetamide (47). However, since the toxicity of thioacetamide S-oxide is enhanced by prior treatment of animals with phenobarbital and is inhibited by pyrazole and cobaltous chloride in noninduced animals and by SKF-525A in phenobarbital-induced animals, it appears that thioacetamide S-oxide must undergo further oxidative metabolism to exert its necrotic effect (47). In a recent study, thiobenzamide-S-oxide was shown to be more hepatotoxic than thiobenzamide, in agreement with the above results (48).

Covalent Binding of Thiono-Sulfur Compounds In Vitro and In Vivo

A number of thiono-sulfur compounds, including parathion (37, 49), carbon disulfide (38, 49), thioacetamide (39), ANTU (27), ANIT (40), and methimazole (50), have been shown to give rise to metabolites which bind covalently to microsomes when incubated in the presence of NADPH. In some cases [carbon disulfide (38, 49), methimazole (50), and ANIT (40)], covalent binding is observed even in the absence of NADPH, suggesting some covalent binding of the parent compound. Covalent binding has been observed in vivo with parathion (36), carbon disulfide (25), thioacetamide (47), ANIT (40), and ANTU (27).

In most cases a good correlation has been obtained between covalent binding in vivo and organ damage after various pretreatments which alter metabolism or after administration of different analogs. Results obtained with ANTU (27) and ANIT (40) will illustrate some of the approaches used. Pretreatment of rats for 5 days with a sublethal dose of ANTU decreased by 60% the covalent binding to lung tissue of ^{35}S from a subsequent challenge dose of ^{35}S-ANTU, and protected against the lung damage and lethality (27). Consistent with this finding, pretreatment of rats with diethyl maleate increased by 150% the covalent binding of ^{35}S to lung tissue and enhanced the lung damage and lethality. Furthermore, less than 10% as much binding from the relatively nontoxic ^{14}C-ANU was observed as from ^{14}C-ANTU. In the case of ANIT (40), pretreatments which decreased the amount of covalent binding such as SKF-525A and cobaltous chloride

also protected against hyperbilirubinemia in rats, whereas phenobarbital increased both covalent binding to liver macromolecules and hyperbilirubinemia.

In some cases the relationship between covalent binding and toxicity is less obvious. Thus pretreatment of rats with ipomeanol for 5 days completely protected against the lung damage and lethality of a challenge dose of ^{35}S-ANTU sufficient to kill all 10 of the nonpretreated animals, but only a 15% decrease in covalent binding of ^{35}S to lung macromolecules was observed (27). With ANIT, pretreatment of rats with 16-α-pregnenolone carbonitrile was found to protect against hyperbilirubinemia, but caused an increase in covalent binding both in vivo and in vitro (40).

Despite the few discrepancies noted, most of the available data strongly suggest a role of active intermediates of thiono-sulfur compounds in mediating their toxicity. Understanding of the mechanisms by which such intermediates may exert their adverse effects requires detailed knowledge of both the structure of the intermediates themselves and of their reactions with macromolecules. The next section will deal with recent developments in this laboratory aimed at elucidating the pathways of activation and biochemical mechanisms of toxicity of representative thiono-sulfur compounds.

PATHWAYS OF METABOLIC ACTIVATION OF SELECTED THIONO-SULFUR COMPOUNDS

Parathion

Parathion is one of a class of phosphorothionate triesters widely used as insecticides. These compounds exert their toxic effects in insects and mammals by inhibiting the enzyme acetylcholinesterase. The phosphorothionates, in general, are relatively poor inhibitors of acetylcholinesterase but are converted by the cytochrome P-450-containing monooxygenase enzyme systems in insects and mammals to the corresponding phosphate triesters which are potent inhibitors of this enzyme.

There are five major products of parathion metabolism. One of these is the corresponding phosphate triester, paraoxon, which is formed in a mixed-function oxidase catalyzed reaction in which the sulfur atom of parathion is replaced by an oxygen atom (51). The paraoxon is subject to hydrolysis by esterases present in various tissues to form diethyl phosphate and *p*-nitrophenol (52). Parathion is not a substrate for these esterases, presumably because the phosphorus atom is not as electrophilic as that in paraoxon.

Parathion is also metabolized to diethyl phosphorothioic acid and *p*-nitrophenol in a reaction requiring a cytochrome P-450-containing monooxygenase enzyme system (53, 54). Studies with $H_2^{18}O$ have indicated that

water, in addition to molecular oxygen and NADPH, is required in this reaction (55). The compound diethyl phosphate plus *p*-nitrophenol can also be formed from parathion in a monooxygenase catalyzed reaction (56).

The first mechanistic studies of the microsomal metabolism of parathion to paraoxon were aimed at establishing (*a*) the initial site of attack of the cytochrome P-450 generated oxygen atom on the parathion molecule, and (*b*) whether the attacking oxygen atom was retained in the final product. The results of model studies of the reactions of parathion and related compounds with peroxy acids (57, 58), and of studies of the stereospecificity of the oxidative desulfuration of such compounds by mouse liver microsomes (59) were consistent with initial attack of oxygen on the sulfur atom. The results of oxygen-18 studies with rabbit liver microsomes and parathion (55) and with rat liver microsomes and dyfonate (60) were consistent with retention of the oxygen atom transferred to parathion by the cytochrome P-450.

From these data, a chemical mechanism for the formation of paraoxon from parathion was proposed (56). This mechanism is shown in Figure 1.

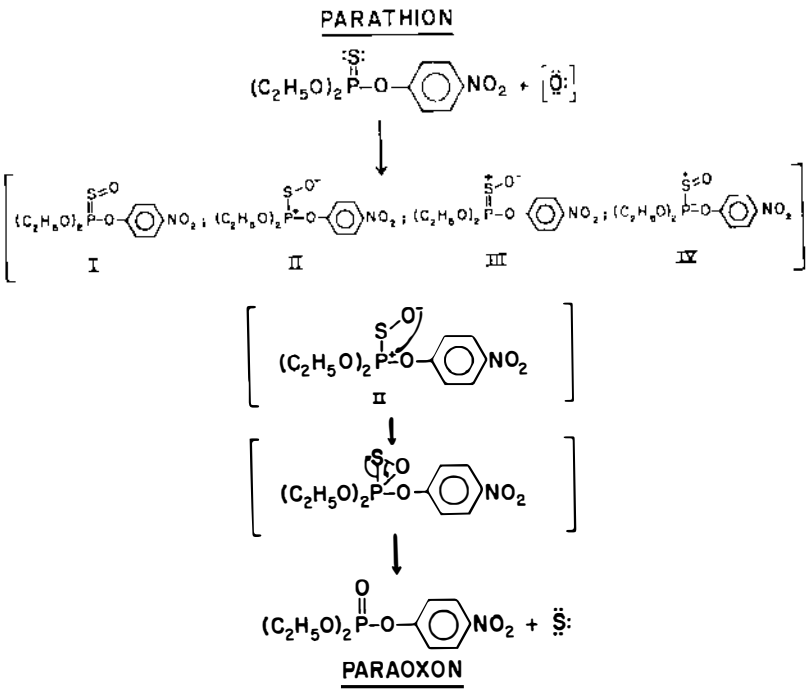

Figure 1 Proposed chemical mechanism of the cytochrome P-450 monooxygenase catalyzed metabolism of parathion to paraoxon.

It was postulated that a singlet oxygen atom generated in a cytochrome P-450 catalyzed reaction is donated to the sulfur atom of parathion to yield a compound analogous to the S-oxide formed in the reaction of peracids with thioketones (61, 62). The attacking singlet oxygen atom is shown in brackets by way of suggesting that it is more likely transferred from cytochrome P-450 to the phosphorothionate sulfur in a concerted reaction. As shown in Figure 1, there are four different structures that may contribute to the resonance stabilization of the resultant S-oxide. It was proposed that one of these resonance forms (perhaps form II) reacts internally to form a cyclic phosphorus-sulfur-oxygen intermediate analogous to the oxathiiran which has been proposed by numerous investigators to be an intermediate in the reaction pathways of various S-oxides (63). This resultant phosphooxathiiran then undergoes a cyclic electron shift with the loss of sulfur, forming paraoxon. The sulfur atom that is released is shown in its singlet form. Although there is at present little evidence to support this, it is probable that if the attacking oxygen atom is in its singlet state the departing sulfur atom may also be in its singlet state.

Atomic sulfur is a highly reactive electrophile which would be expected to bind readily to nucleophiles near the site of its release. The thiono-sulfur group of parathion has been found to be covalently bound to tissue macromolecules following administration of [^{35}S] parathion in vivo (36) and on incubation with hepatic microsomes in vitro (37, 49). When double-labeled parathion (^{32}P, ^{35}S) was incubated in the absence of NADPH with microsomes isolated from the livers of phenobarbital-pretreated rats, only a trace of radioactivity could be found bound to the microsomes (36). However, when the incubation was carried out in the presence of NADPH, a substantial amount of sulfur became covalently bound to the microsomes. A small but significant amount of the phosphorus-containing portion of the parathion molecule was also covalently bound to the microsomes. These results clearly indicated that the majority of the sulfur bound to the microsomes was free of the phosphorus-containing portion of the molecule and thus must be atomic sulfur released in the metabolism of parathion to paraoxon. This is further substantiated by the finding that the amount of sulfur bound under these conditions is equivalent to the amount of paraoxon formed in the incubation (37). When an amount of paraoxon which was approximately five times the amount that would be expected to be formed in the incubation containing doubly labeled parathion was incubated with an equal aliquot of the same preparation of microsomes, a much smaller amount of ^{32}P was bound using [^{32}P] paraoxon than was bound using the doubly labeled parathion. Thus the binding of ^{32}P in a reaction of paraoxon with nucleophilic sites on the endoplasmic reticulum is responsible for only a small portion of the total binding of ^{32}P seen using

doubly labeled parathion. It appears that the greater portion of the ^{32}P binding in the incubation using doubly labeled parathion is likely the result of the reaction of one or more of the intermediate S-oxides shown in Figure 1 with nucleophiles on the endoplasmic reticulum. In this context it should be noted that the incubation of the other phosphorus-containing metabolites of parathion with microsomes in the presence of NADPH does not lead to the binding of any radioactivity.

The binding of sulfur and/or an activated intermediate of the phosphorus-containing portion of the parathion molecule to the endoplasmic reticulum leads to a decrease in the amount of cytochrome P-450 detectable as its carbon monoxide complex and to a decrease in the rate of metabolism of substrates such as benzphetamine (37, 49, 64). Paraoxon, or any other of the isolatable metabolites of parathion, do not decrease the amount of cytochrome P-450 or inhibit the ability of microsomes to metabolize substrates such as benzphetamine (37).

The metabolism of parathion by purified reconstituted mixed-function oxidase enzyme systems isolated from the livers of phenobarbital-pretreated rabbits (56) and rats (65) has also been examined. All three of the major phosphorus-containing metabolites of parathion can be formed by what appears to be a single species of cytochrome P-450. These data and those from the studies using a peroxy acid model system (58) suggest that the mixed-function oxidase enzyme system is only involved in the addition of an oxygen atom to the phosphorothionate sulfur (Figure 1) and that the various products are formed nonenzymatically from a common intermediate. It is believed that this intermediate is one or more of the resonance forms of the intermediate S-oxide shown in Figure 1.

Recent efforts in this laboratory have employed the reconstituted monooxygenase system from rat liver microsomes to elucidate the mechanism by which parathion causes a loss of cytochrome P-450 detectable as its carbon monoxide complex and a loss of cytochrome P-450-dependent monooxygenase activity. Inhibition of the cytochrome P-450 is accompanied by covalent binding of sulfur to the enzyme, and occurs only in the presence of a complete system, i.e. under conditions where metabolism of parathion takes place (65). The sulfur which becomes covalently bound to the P-450 appears to be free of the rest of the parathion molecule, based on the almost negligible amount of covalent binding observed when [^{14}C ethyl] parathion rather than [^{35}S] parathion is used (65).

Experiments using a partially purified antibody to the major phenobarbital-induced form of rat liver cytochrome P-450 indicated that P-450 was the predominant if not sole protein attacked by atomic sulfur. Similar results were obtained upon SDS gel electrophoresis of the [^{35}S] labeled proteins of the reconstituted system (65). The SDS gel electrophoresis also revealed

that metabolism of parathion by a reconstituted system leads to the formation of high molecular weight aggregates. The aggregates could be dissociated by treatment with β-mercaptoethanol, dithiothereitol, or cyanide. The CN^- treatment released approximately 50% of the covalently bound sulfur as $^{35}SCN^-$, suggesting the presence of hydrodisulfide linkages (R-S-^{35}SH), apparently formed by attack of [^{35}S] on cysteine residues in the cytochrome P-450. Very recently dithiothreitol treatment was found to release 75% of the [^{35}S] bound to the proteins of the reconstituted system (66). The remaining ^{35}S was found to be stable to treatment with performic acid and to acid hydrolysis, and to be distributed among at least three different amino acids. Probable mechanisms for the formation of such amino acid adducts are carbon-hydrogen insertion (a known reaction of singlet atomic sulfur), or addition of atomic sulfur across a double bond (65). There is no evidence however, that modification of these three amino acids plays any direct role in the loss of cytochrome P-450 or monooxygenase activity (66).

Metabolism of parathion was found to result in a considerable loss of heme from the cytochrome P-450 of the reconstituted system (66). The heme loss, which could not be prevented with catalase, was sufficient to account for most of the loss of cytochrome P-450 detectable as its carbon monoxide complex. However, the heme loss could account for only 50% of the loss of P-450-dependent monooxygenase activity, suggesting that alterations of the protein moiety were also in part responsible for the inactivation of the enzyme. However, such modifications do not appear to involve simple derivatization of essential amino acid residues in the P-450, since removal of 75% of the bound radioactivity regenerates no enzymatic activity. At present it is still unclear exactly what the critical modification of the protein moiety is, although it appears likely that the aggregation accompanying the binding of atomic sulfur to cysteine residues plays some role.

Carbon Disulfide

Although the neurological effects of carbon disulfide may be the most prevalent from the standpoint of human exposure, most experimental research using laboratory animals has focused on the effects of carbon disulfide exposure on the liver. A marked decline in the level of cytochrome P-450 and cytochrome P-450-dependent monooxygenase activity measured in vitro is detectable in rats administered CS_2 orally or by i.p. injection (5–7, 25, 38, 49, 67) or exposed by inhalation (68, 69). The administration of CS_2 to rats pretreated with phenobarbital causes an even greater decrease in P-450 levels and associated enzymatic activities (5, 7) and also produces moderate to severe centrilobular hydropic degeneration of the liver (5, 7,

41, 42). Prior administration of SKF-525A to phenobarbital pretreated animals decreases the liver damage caused by subsequent exposure to CS_2, suggesting that the hepatic toxicity of CS_2 is mediated by toxic metabolites produced by the cytochrome P-450-dependent monooxygenase system (6, 41). This was substantiated by the findings that phenobarbital increases the in vivo binding of both [^{14}C] and [^{35}S] from CS_2 to liver microsomes (25) and that there is a correlation between the amount of a dose of [^{14}C]CS_2 excreted as [^{14}C]CO_2 and the degree of liver damage (6).

Recent efforts have been concentrated on elucidating the nature of the metabolites responsible for the inhibition of cytochrome P-450 and the liver damage caused by carbon disulfide. It was speculated that CS_2 might be metabolized to COS and then CO_2 in two sequential steps analogous to the conversion of parathion to paraoxon, and leading to the release and covalent binding of one or both of the sulfur atoms. In vitro experiments with microsomes from untreated and phenobarbital-treated rats and carbon disulfide labeled on either the carbon or sulfur atoms were carried out to test this hypothesis (38). In the absence of NADPH equal amounts of [^{35}S] and [^{14}C] became bound to the microsomal proteins, suggesting the direct reactions of CS_2 with amino or sulfhydryl groups. In the presence of NADPH [^{35}S] binding to control microsomes was increased twofold and to phenobarbital microsomes sixfold. The stimulation by NADPH of the binding of sulfur from CS_2 to microsomes suggested that CS_2 was a substrate for cytochrome P-450. This was supported by the finding that the amount of sulfur bound in the presence of NADPH was inhibited by carbon monoxide (38) or SKF-525A (67).

Whereas in the phenobarbital microsomes NADPH led to a sixfold increase in [^{35}S] binding, only a twofold increase in [^{14}C] binding was observed (38). This indicated that the majority of the [^{35}S] bound to the microsomal proteins was free of the carbon atom. Experiments where the [^{35}S] labeled microsomes were treated with CN^- indicated that approximately 50% of the sulfur which had become bound to the microsomal proteins during the metabolism of CS_2 was in the form of a hydrodisulfide (70), analogous to the results obtained with parathion, which were described in the previous section.

The results described up to now suggest a high degree of similarity between the metabolism of parathion and of carbon disulfide. However, the most recent work in this laboratory indicates that the two thiono-sulfur compounds may be metabolized by quite different pathways.

The first finding that cast doubt on the hypothesis that cytochrome P-450 is the major enzyme responsible for converting CS_2 to COS and, in turn, to CO_2 was that most of the rat hepatic metabolic activity towards COS was concentrated in the cytosol, although some NADPH-dependent activity

was found in the microsomes (71). The cytosolic activity could be attributed to the enzyme carbonic anhydrase since it was inhibited by the inhibitor acetazolamide, and since COS was shown to be a substrate for the bovine erythrocyte enzyme. The carbonic anhydrase activity in rat liver measured with CO_2 as the substrate was more than adequate to account for the rates of COS metabolism observed. Carbonic anhydrase was proposed to convert COS to monothiocarbonic acid (H_2CO_2S), which breaks down to CO_2 and HS^-. On this basis it was suggested that production of HS^- from COS by carbonic anhydrase might be the mechanism of toxicity of COS. A subsequent study in rats (72) demonstrated that COS is converted to hydrogen sulfide in vivo, and that the blood levels of HS^- and the toxicity of COS are decreased by pretreatment with acetazolamide. Acetazolamide had no effect on HS^- toxicity per se, whereas treatment with sodium nitrite, which protects against HS^- toxicity, also protected against COS toxicity. These results are all consistent with a key role of HS^- in the central respiratory arrest caused by COS. Whether HS^- plays a role in the acute or chronic toxicity of CS_2 remains unclear.

Recent findings concerning the mechanism of conversion of CS_2 to COS also demonstrate important differences between CS_2 and parathion metabolism. Thus, whereas oxygen-18 studies with parathion indicate that the oxygen which is transferred from cytochrome P-450 is retained in the paraoxon molecule (55), studies with $H_2^{18}O$ and carbon disulfide suggested that all the oxygen in COS comes from water (C. P. Chengelis and R. A. Neal, unpublished observations). These data are not consistent with an oxithirane intermediate of CS_2 of the kind proposed with parathion (Figure 1), but rather suggest attack of water on the intermediate S-oxide ($S{=}C{=}S^+{-}O^-$) formed in a P-450 catalyzed reaction. By analogy with parathion it would appear that binding of sulfur released from CS_2 to cytochrome P-450 is the key event in inactivating the enzyme, whereas it appears likely that binding of the S-oxide to other nucleophiles is involved in producing liver damage.

Thioacetamide

Thioacetamide and a metabolite, thioacetamide S-oxide, both produce centrilobular hepatic necrosis in male Sprague-Dawley rats when administered at doses of 1.25–5.0 mmol/kg i.p. (10, 47). Thioacetamide S-oxide produces more severe necrosis 24 hours after administration than does an equimolar dose of thioacetamide; additionally, histological changes in liver cells induced by thioacetamide S-oxide are detected at earlier times than corresponding changes induced by thioacetamide. The toxicity of both compounds is enhanced by prior treatment of the rats with phenobarbital and is inhibited by pyrazole and cobaltous chloride in noninduced animals

and by SKF-525A in phenobarbital-induced animals, suggesting that further metabolic activation is required for either compound to exert its necrotic effect (10).

Upon administration of [^{3}H] thioacetamide in vivo to rats, maximal covalent binding to liver molecules is observed after 6 hours, with necrosis being initially seen after approximately 12 hours (47). With [^{3}H] thioacetamide S-oxide, the maximal covalent binding, which is about twice that seen with an equimolar dose of thioacetamide, occurs at approximately 3 hours, with necrosis being detectable at about 7–8 hours. In isolated hepatocytes approximately six times as much binding of [^{3}H] from thioacetamide S-oxide as from thioacetamide is observed (M. J. Gudzinowicz and R. A. Neal, unpublished observations). In addition, incubation of hepatocytes with thioacetamide S-oxide leads to a significant decrease in the viability of the cells, as measured by trypan blue exclusion, at about 6 hours, with only about 20% survival at 10 hours. On the other hand, with thioacetamide, relatively little decrease in viability is seen at 10 hours. The results of both the in vivo and in vitro studies strongly suggest that a reactive metabolite of thioacetamide S-oxide may be responsible for hepatic necrosis.

Recent efforts have been directed towards elucidating the nature of the metabolite which becomes covalently bound to liver macromolecules in vivo (47). In one study thioacetamide S-oxide radiolabeled in various portions of the molecule was administered to rats, and the nature of the radioactivity covalently bound to liver macromolecules was examined (47). The results of these experiments showed that the amount of binding of [^{3}H] methyl thioacetamide S-oxide and [^{14}C] carbonyl thioacetamide S-oxide was for all intents and purposes equal. However, little or no covalent binding of [^{35}S] from [^{35}S] thioacetamide S-oxide was observed. Thus the bound metabolite appeared to contain the methyl group and the carbonyl carbon but not the sulfur atom.

In order to elucidate the structure of the covalently bound adduct, liver cytosolic proteins labeled in vivo by administration of [^{3}H] or [^{14}C] thioacetamide S-oxide to rats were digested with pronase (M. C. Dyroff and R. A. Neal, unpublished observations). By chromatography on Dowex 50 and on a Beckman amino acid analyzer an amino acid adduct accounting for approximately 70% of the bound radioactivity could be isolated. This adduct was identified as *N*-ε-acetyl-L-lysine by cochromatography with a standard on the amino acid analyzer and by gas chromatography-mass spectrometry. Thus either during the process of binding to protein or during the isolation procedure the nitrogen atom from thioacetamide S-oxide appears to be lost.

In contrast to other thiono-sulfur compounds examined, thioacetamide does not cause a decrease in the level of cytochrome P-450 detectable as its carbon monoxide complex or to a decrease in cytochrome P-450 monooxygenase activity when incubated with hepatic microsomes in vitro (7). The lack of inhibition of this enzyme by thioacetamide could be because thioacetamide, in contrast to other thiono-sulfur compounds examined, forms a stable S-oxide. Thus, the rearrangement of the thioacetamide S-oxide with the release and covalent binding to cytochrome P-450 of atomic sulfur does not occur.

An alternative explanation, however, is that thioacetamide and its S-oxide are not primarily metabolized by cytochrome P-450. Thus, although the major phenobarbital-induced form of cytochrome P-450 is capable of metabolizing thioacetamide to its S-oxide, the rate is much slower than that observed with intact phenobarbital microsomes, and a partially purified antibody to the cytochrome P-450 inhibits at most 25% of the microsomal metabolism (39). Although the antibody is more effective in inhibiting thioacetamide S-oxide metabolism in phenobarbital microsomes, the purified enzyme does not appear to metabolize the compound. Use of the purified enzyme or the antibody could not assess the role of cytochrome P-450 in microsomes from noninduced animals, which do, however, exhibit necrosis.

A number of sulfur compounds including thioureas and thioamides are known to be good substrates for the microsomal flavin-containing monooxygenase from hog liver (73), which is also present in rat liver (74). The purified hog liver enzyme converts thioamides to mono- and di-S-oxygenated derivatives. Therefore the role of this enzyme in producing toxic metabolites from thioacetamide and its S-oxide was considered in a recent investigation (M. J. Gudzinowicz and R. A. Neal, unpublished observations).

SUMMARY

Thiono-sulfur-containing compounds cause a wide variety of toxic effects in mammals. These toxic effects of thiono-sulfur-containing compounds appear to be at least partially the result of their metabolism to reactive intermediates by the cytochrome P-450-containing monooxygenase enzyme systems. Covalent binding of (atomic) sulfur released in the cytochrome P-450 monooxygenase catalyzed metabolism of certain thiono-sulfur compounds appears to be responsible for the inhibition of monooxygenase activity and the loss of cytochrome P-450 seen on administration of these thiono-sulfur compounds in vivo or incubation with cytochrome P-450 monooxygenase enzymes in vitro. Liver necrosis and perhaps the induction

of lung edema and neoplasia as well as other effects of thiono-sulfur-containing compounds are more likely the result of the covalent binding of the electrophilic S-oxides or S-dioxides or carbene derivatives of these S-oxides and S-dioxides to tissue macromolecules. The rationale for implicating metabolites of thiono-sulfur compounds other than atomic sulfur in these effects derives from the experiments with thioacetamide and the fact that atomic sulfur is highly reactive and appears to bind predominantly or exclusively to cytochrome P-450. It is difficult to rationalize why binding to and inhibition of cytochrome P-450 would lead to the production of, for example, liver necrosis.

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