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The Role of Biotransformation in Chemical-Induced Liver Injury

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The role of drug metabolism in chemical-induced liver injury is reviewed. Parameters for studying the formation of chemically reactive metabolites are discussed and the factors that alter the formation and covalent binding of reactive metabolites are selectively emphasized. Some of the experimental work that led to these concepts is discussed, especially the chemical toxicology of the hepatic injury produced by acetaminophen, bromobenzene, furosemide, isoniazid and iproniazid.

An important result of drug metabolism studies in recent years has been the realization that many foreign compounds are metabolized by the liver and certain other tissues to potent alkylating, arylating, or free-radical intermediates (*1-12*). Such studies demonstrate how chemically stable compounds can produce serious tissue lesions in man and experimental animals, including neoplasia, hepatic and renal necrosis, bone marrow aplasia, and other injuries. These studies frequently demonstrate a role for sulfhydryl containing compounds, particularly glutathione, in protecting tissues from such toxic reactions.

Many of the initial concepts of metabolic activation were developed during studies of chemical carcinogenesis; the work of the Millers in the United States (*1,2*) and of Magee and co-workers in England (*3*) has been especially illuminating. The realization that the enzyme pathways responsible for the metabolic activation of carcinogens are the same microsomal mixed-function oxidases that metabolize most drugs and other xenobiotics led to the concept that drug-induced tissue lesions might also be mediated through the covalent binding of reactive metabolites (*6-11*). The lack of reactivity of most chemically stable drugs and the frequent localization of tissue damage only in those organs or to those animal species having the necessary drug-metabolizing enzymes supported this view.

Obviously, most drugs and foreign compounds that enter the body are converted to chemically inert metabolites that are readily excreted into urine, bile, or air. Thus, it has become important to identify when toxicities are mediated by chemically reactive metabolites and when they are caused by chemically inert metabolites. Because the latter produce their effects by combining reversibly with receptor sites, their toxicologic activity usually can be evaluated simply by measuring the concentration of the metabolite in body fluids (*13*). When the response is tissue damage caused by the covalent binding of chemically inert metabolites to tissue macromolecules, however, rarely can the relationship between tissue levels of the metabolite and the severity of the lesion be determined. Indeed, highly reactive metabolites may exist for only a few seconds or less and thus will never accumulate in body fluids.

Parameters for Studying Reactive Metabolites

How then can one readily determine the formation of such chemically unstable and reactive metabolites? It seemed possible that there might

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be a relationship between the severity of the lesion and the amount of covalently bound metabolite for any particular drug or chemical. The covalent binding of the reactive metabolite could then be used as an index of the formation of the metabolite. Furthermore, this parameter might well be the most reliable estimate of the availability of the metabolite *in situ* for causing tissue damage, since much of the metabolite often decomposes or is further metabolized before it can be isolated in body fluids or urine. Thus, one approach to the problem would be to determine whether radiolabeled drugs administered to animals over a wide dose range are covalently bound to macromolecules in target tissues that subsequently become necrotic.

This approach has been used to implicate toxic metabolites as mediators of the hepatic necrosis produced in animals by commonly administered drugs, such as acetaminophen (paracetamol), acetanilide, phenacetin, furosemide, isoniazid-acetylisoniazid, and iproniazid, and by chemical model toxins, such as halobenzenes, furans, thiophenes and hydrazines (*6-11*). These hepatotoxic compounds covalently bind to tissue marcromolecules when administered *in vivo*. Since they are chemically stable substances, the finding of a covalent linkage with macromolecules in their target tissue, the liver, indicates that they are converted in the body to chemically reactive intermediates. Moreover, autoradiograms show that the binding occurs preferentially in the necrotic areas of the liver. Covalent binding can also be measured quantitatively by extraction of tissue proteins with organic solvents or by isolation of the radiolabeled material bound to single amino acids. Pretreatment of animals with inducers of drug metabolism, such as phenobarbital, or with inhibitors of drug metabolism, such as piperonyl butoxide, cobalt chloride or α-naphthylisothiocyanate (ANIT), similarly alter the rate of metabolism of the hepatotoxins, the extent of hepatic binding of radiolabeled metabolites and the severity of hepatic necrosis.

Recent work has extended these concepts to include the acute renal tubular necrosis produced by acetaminophen and the antibiotic drug, cephaloridine (Loridine)(*10, 11*). Preliminary evidence suggests that chemically reactive metabolites of several aminophenol, furan, and thiophene model nephrotoxins are responsible for the renal damage caused by the compounds (*10,11*).

Factors that Alter the Formation and Covalent Binding of Reactive Metabolites

In some instances, factors that accelerate or decelerate drug metabolism may affect the formation and covalent binding of reactive metabolites in seemingly unpredictable ways. The reason for many of these results becomes clear after perusing the kinetics of covalent binding *in vivo* (Table 1) (*14*). If the conjugates between the macromolecules and the reactive metabolites are not rapidly metabolized, then the amount of covalently bound metabolites will accumulate until all of the drug is metabolized. But it is important to realize that the proportion of the dose which becomes convalently bound is not always dependent on the rate at which the drug is converted to the reactive metabolite. For example, if all of the parent drug were converted to the reactive metabolite and all of the chemically reactive metabolite became covalently bound to tissue macromolecules, then all of the parent drug would be covalently bound regardless of the rate at which the drug is converted to its chemically reactive metabolite or the rate at which the chemically reactive metabolite combines with the tissue macromolecules. Thus, inducers and inhibitors of drug-metabolizing enzymes do not result in changes in covalent binding simply because they change the rate of drug metabolism. Instead they alter the amount of covalent binding because these changes in rates result in changes in the proportion of the dose that becomes converted to the chemically reactive metabolite or in the fraction of the reactive metabolite that be-

Table 1. Important kinetic factors that must be considered when evaluating pharmacologic and toxicologic effects of chemically reactive metabolites.

A. Do inducers or inhibitors alter the relative proportion of the dose that is converted to the active metabolite?
B. Do inducers or inhibitors alter the relative proportion of the reactive metabolite that becomes covalently bound?
C. Do other substances change the tissue levels of cosubstrates used in conjugation reactions with the reactive metabolite?
D. Do high doses of the hepatotoxin itself lead to depletion of cosubstrates for conjugation reactions with the reactive metabolite?
E. Are chemically reactive metabolites formed in different tissues?
F. Do chemically reactive metabolites leave the tissues in which they are formed?

comes covalently bound or both. Therefore, it is not the rate of drug metabolism *per se*, but the pattern of metabolism that determines the magnitude of covalent binding.

Whether a given amount of covalently bound metabolite results in toxicity depends on a host of factors. If the chemically reactive metabolite reacted only with vitally important macromolecules, then there might be a direct relationship between the amount of covalently bound metabolite and the severity of the lesion regardless of the tissue in which the covalent binding occurred. It seems likely, however, that the covalent binding to macromolecules would be rather indiscriminate and that only a portion of the total covalently bound metabolite would be bound to vitally important macromolecules. Indeed, in some instances the covalent binding to the vitally important components of the cell may not even be a part of the material being assayed. For example, CCl_4 is thought to exert its toxic effects by a peroxidative interaction between its reactive metabolite and lipids, but this interaction would not be measured by the methods used to measure the covalent binding of the reactive metabolite with tissue proteins. According to this view, the amount of covalently bound metabolite required to cause a specific kind of tissue damage, such as necrosis, will vary with the foreign compound and the tissue. In order to relate the formation of reactive metabolites with any given toxicity, therefore, one must consider at least four kinetic determinants: (1) the proportion of the dose of the toxicant that is converted to a chemically reactive metabolite; (2) the proportion of the reactive metabolite that becomes covalently bound to cellular components; (3) the proportion of covalently bound metabolite that is attached to vitally important cellular components; and (4) the proportion of such covalently bound metabolite that cannot be replaced or repaired rapidly by the cell. Obviously, the values of covalently bound metabolite (3) and (4) will vary with the toxicant, the tissue in which the covalent binding occurs, and the mechanism of toxicity. When the product of (3) and (4) is small, toxicity may not occur even though considerable amounts of covalently bound metabolites are found in tissues. Conversely, when the product of (3) and (4) is high, toxicity may occur when only small amounts of covalently bound metabolite are found in tissues. Thus, the importance of numerical values of (3) and (4) are virtually impossible to estimate at this time and it is apparent that measurement of covalent binding alone cannot be used to predict whether a given compound will cause a given kind of toxicity. Only by correlating changes in the amount of covalently bound metabolite with changes in the incidence and severity of the toxicity can one determine whether the injury is mediated through the formation of a chemically reactive metabolite.

Experimental Work Which Led to Above Concepts

The necessity of examining the covalent binding of reactive metabolites *in vivo* simultaneously with the determination of tissue injury and the pattern of urinary metabolites is evident from the following studies.

Acetaminophen

In therapeutic doses, acetaminophen is among the safest of all minor analgesics, but in large overdoses it can produce fatal hepatic necrosis in humans (*15*) and experimental animals (*16–18*). In mice, it does not cause centrilobular necrosis in liver unless the dose is greater than about 300 mg/kg (*17*). Pretreatment of mice or rats with phenobarbital or 3-methylcholanthrene, which increases the metabolism of many drugs, markedly increases the incidence and severity of the necrosis, whereas prior administration of piperonyl butoxide or cobaltous chloride, which inhibits the metabolism of many drugs, decreases the incidence and severity of the toxicity (*17*). Although these data suggested the possibility that the necrosis was caused by a toxic metabolite, they could not reveal whether the metabolite was chemically reactive or inert.

Studies with radiolabeled acetaminophen showed that acetaminophen is converted to a chemically reactive metabolite that becomes convalently bound to liver protein (*19*). Since pretreatment of mice with phenobarbital increased the amount of covalent binding to liver protein, whereas prior administration of piperonyl butoxide or cobaltous chloride decreased it, the liver necrosis was apparently caused by the formation of a chemically reactive metabolite (*19*).

After subtoxic doses of acetaminophen, there was very little covalent binding of radiolabel to liver protein. As the dose was increased to the toxic range the proportion of the dose that became covalently bound markedly increased, indicating that the rate of metabolism of acetaminophen or its reactive metabolite no longer

followed first-order kinetics after toxic doses (*20*). The reason for the dose threshold for the covalent binding and toxicity of acetaminophen became clear when it was discovered that the concentration of glutathione was rapidly decreased after the administration of toxic doses of the drug. The principal pathways of acetaminophen metabolism are shown in Figure 1. A small proportion (about 13% in mice) of a nontoxic dose of acetaminophen is converted to a reactive metabolite that rapidly combines with glutathione to form a conjugate that ultimately is excreted as a mercapturic acid. After the glutathione in the liver is decreased, however, the steady-state concentration of the reactive metabolite markedly increases and reacts with protein. In accord with this view, prior administration of diethyl maleate, which decreases the glutathione concentration in liver without causing liver necrosis, markedly potentiates the liver damage caused by acetaminophen (*20*). Moreover, diets that lower the concentration of glutathione in liver enhance the toxicity of acetaminophen (*21*). On the other hand, the administration of cysteine and cysteamine in mice (*6, 20, 22*) or methionine in rats (*21*), which presumably react either directly or indirectly with the reactive metabolite, prevented the liver necrosis.

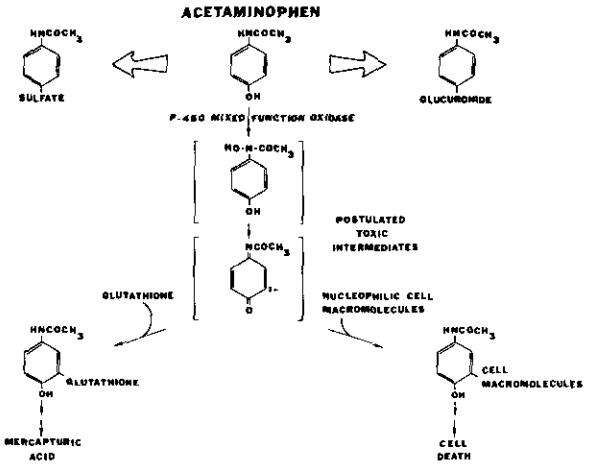

FIGURE 1. Pathways of acetaminophen metabolism.

Studies *in vitro* revealed that pretreatment of mice with phenobarbital increases the rate of formation of the reactive metabolite (*23*). However, this pretreatment does not significantly alter the biological half-life of acetaminophen in mice (*17*), presumably because phenobarbital does not induce the enzymes that convert the drug to its glucuronide and sulfate conjugates in this strain of mice. By contrast, prior administration of piperonyl butoxide, which inhibits the covalent binding *in vitro* (*23*), increased the half-life of acetaminophen *in vivo* (*17*).

In hamsters, acetaminophen causes liver necrosis in doses as low as 150 mg/kg (*18, 24*). But unlike the effects of phenobarbital in mice, the prior administration of phenobarbital to hamsters decreases both the covalent binding of radiolabeled acetaminophen and the liver necrosis caused by the drug (*24*). The reason for this species difference became clear when it was discovered that phenobarbital pretreatment of hamsters did not increase the rate of formation of the reactive metabolite by liver microsomes, but markedly decreased the biological half-life of acetaminophen *in vivo* by increasing glucuronidation of the drug (*24*). These findings thus demonstrate that a pretreatment may have a profound effect on the formation of reactive metabolites and on toxicity even though it does not markedly alter the activities of the enzymes that catalyze either the formation or inactivation of the reactive metabolite.

The effects of 3-methylcholanthrene, diethyl maleate, piperonyl butoxide, and cobaltous chloride on acetaminophen-induced liver necrosis in hamsters (*24*) were similar to those in rats (*17*). The prior administration of 3-methylcholanthrene to hamsters increased the formation of the reactive metabolite of acetaminophen by liver microsomes and accelarated the depletion of liver glutathione, but had little effect on the biological half-life of acetaminophen *in vivo*. Accordingly, 3-methylcholanthrene increased both the covalent binding of radiolabeled acetaminophen to liver proteins *in vivo* and the severity of the liver necrosis. By contrast, piperonyl butoxide decreased the formation of the reactive metabolite by hamster liver microsomes, decelerated the depletion of liver glutathione *in vivo* and increased the biological half-life of acetaminophen. Despite the increase in the biological half-life of the drug, piperonyl butoxide still decreased both the covalent biding of radiolabeled acetaminophen and the severity of the liver necrosis.

In contrast to its effects in mice and hamsters, acetaminophen causes only minor liver damage in rats at doses as large as 1500 mg/kg (*16-18, 21*), in guinea pigs in doses as large as 500 mg/kg and in rabbits in doses as large as 750 mg/kg (*18*). Studies with liver microsomes revealed that the

lack of toxicity in these animal species may be partially due to low V_{max} values for the formation of the reactive metabolite by those from rabbits and guinea pigs and an unusally high K_m (acetaminophen) value for its formation by those from rats (*18,23*). In accord with these findings the concentration of glutathione in liver after the administration of acetaminophen (300 mg/kg, IP) was only slightly decreased in rats and guinea pigs (*18,23*).

Studies on the distribution of the urinary metabolites of acetaminophen in different animal species also illustrate the point that species differences in the amount of a metabolite formed from a reactive metabolite (e.g., a glutathione conjugate) do not always parallel the species differences in the amount of covalently bound metabolite. At low nontoxic doses of acetaminophen virtually all of the reactive metabolite is converted to its mercapturic acid (Fig. 2) (*25*). Thus, the fraction of the dose of acetaminophen that is excreted into urine as the mercapturic acid under these conditions may be used as an indirect estimate of the species difference in the fraction of the dose that is converted to the reactive metabolite *in vivo*. When low, nontoxic doses were administered (50 mg/kg, IP) about 4% of the dose was excreted as the mercapturic acid in rats and about 13-15% was excreted as the mercapturic acid in mice and hamsters (Table 2). Thus, species differences in mercapturic acid excretion at these low doses of acetaminophen parallel the species differences in the covalent binding of the reactive metabolite and the liver necrosis caused by high doses of acetaminophen. When a large dose of acetaminophen (400 mg/kg, IP) was administered, however, the glutathione concentrations in liver were markedly decreased in mice and hamsters, which resulted in considerable amounts of covalently bound reactive metabolite, and thus the proportion of the dose of acetaminophen excreted as the mercapturic acid was decreased to about 6.5% in mice and about 10% in hamsters (Table 2). In contrast, when this large dose of acetaminophen was administered to rats, the glutathione concentration in liver was changed only slightly (Table 2), and thus the proportion of the dose excreted as the mercapturic acid was the same as that obtained with the nontoxic dose (about 4%). Thus, the fraction of the large dose excreted as the mercapturic acid was similar in rats, mice, and hamsters, but this dose of acetaminophen caused liver necrosis only in mice and hamsters.

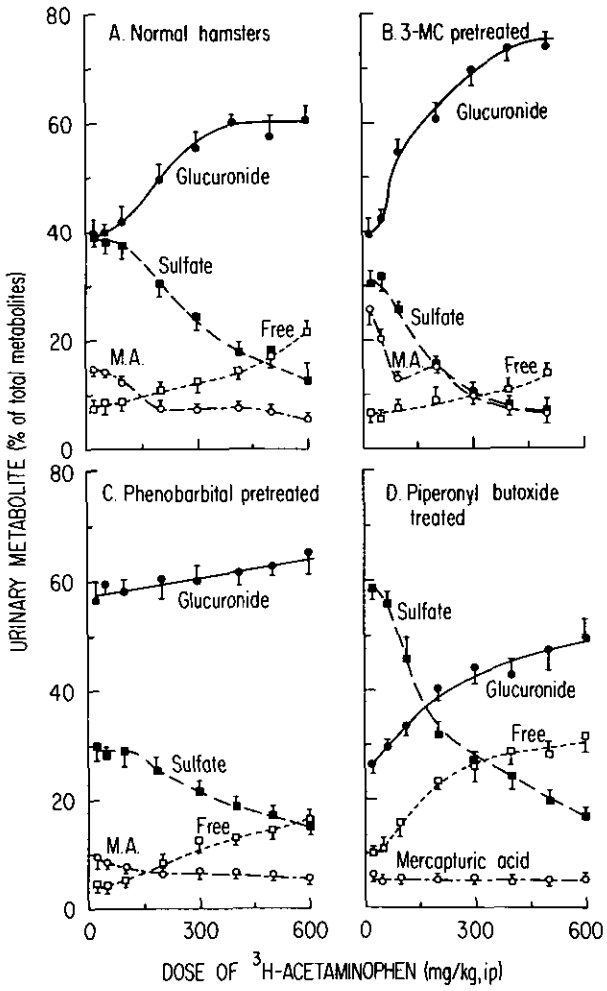

FIGURE 2. Effect of dose on the metabolic disposition of ^{3}H-acetaminophen expressed as percentage of urinary radioactivity (*25*): (*A*) normal hamsters; (*B*) 3-methylcholanthrene-pretreated hamsters; (*C*) phenobarbital-pretreated hamsters; (*D*) piperonyl butoxide-treated hamsters; MS = acetaminophen mercapturic acid. Values are means ± SEM of four observations.

Table 2. Effect of dose of acetaminophen on the urinary excretion of its mercapturic acid in various animal species.[a]

Species	Mercapturic acid, % of dose			Liver necrosis (400 mg/kg dose)
	50 mg/kg	400 mg/kg	650 mg/kg	
Rat	3.6	3.8	3.8	No[b]
Mouse	13.3	6.5	—	Yes
Hamster	14.2	9.6	5.6	Yes

[a] Data calculated from references (*17,18,24,25*).
[b] At doses of 1000 mg/kg, acetaminophen caused liver necrosis in 2% of the animals (*17,18*).

The point is further illustrated by the effects of various treatments on the excretion of the acetaminophen mercapturic acid in hamsters. Pretreatment with 3-methylcholanthrene increased the excretion of the mercapturic acid after the administration of a low, nontoxic dose of acetaminophen (25 mg/kg) to about 26% of the dose, whereas the prior administration of piperonyl butoxide decreased it to about 5% (Fig. 2). By contrast, the proportion of the dose excreted as the mercapturic acid after the injection of a high dose (300 mg/kg) in hamsters pretreated with 3-methylcholanthrene was only double that excreted in hamsters pretreated with piperonyl butoxide, because the proportion of the dose of acetaminophen excreted as the mercapturic acid was decreased as the dose of the drug was increased in 3-methylcholanthrene pretreated hamsters but was not not changed in hamsters given piperonyl butoxide (Table 3). But at 300 mg/kg, acetaminophen caused massive necrosis in all hamsters pretreated with 3-methylcholanthrene and only slight damage in those pretreated with piperonyl butoxide (*24*).

Table 3. Effect of treatments on the excretion of acetaminophen mercapturic acid after different doses of the drug.[a]

Pretreatment	Mercapturic acid, % of dose: 25 mg/kg	Mercapturic acid, % of dose: 300 mg/kg	Liver necrosis
Control	14.4	7.0	Moderate
3-Methylcholanthrene	26.3	10.7	Massive
Piperonyl butoxide	5.0	4.3	Minimal

[a] Data taken from Potter et al. (*24*) and Jollow et al. (*25*).

Bromobenzene

Other principles may be illustrated with studies on the hepatic necrosis induced by halogenated hydrocarbons such as bromobenzene. Although the urinary metabolites of bromobenzene were identified many years ago (*26,27*), recent studies have revealed that nearly all of the bromobenzene administered to animals is converted to its chemically reactive metabolite, bromobenzene 3,4-epoxide, by a cytochrome P-450 enzyme localized mainly in the endoplasmic reticulum of liver (Fig. 3) (*28–30*). Some of the epoxide rearranges nonenzymatically to form 4-bromophenol. Some is converted to a dihydrodiol by an epoxide hydratase in liver endoplasmic reticulum, the dihydrodiol in turn is dehydrogenated to 4-bromocatechol by an enzyme in the soluble fraction of liver. But about 70% of the epoxide formed in rats receiving a nontoxic dose of bromobenzene (50 μmole/kg) is converted to a glutathione conjugate by one or more glutathione transferases in the soluble fraction of liver; the conjugate is then converted to a mercapturic acid that is excreted into urine (*29,30*). Thus, liver injury depends on the relative rates at which bromobenzene 3,4-epoxide becomes covalently bound to tissue macromolecules or is converted to 4-bromophenol,3,4-dihydro-3,4-dihydroxybromobenzene, and 3,4-dihydro-3-hydroxy-4-glutathionylbromobenzene (Fig. 3).

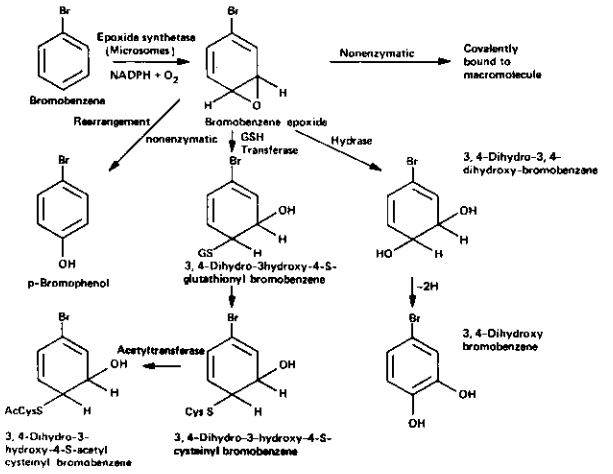

FIGURE 3. Pathways of bromobenzene metabolism.

As with acetaminophen, there is a dose threshold for the covalent binding of radiolabeled bromobenzene. The proportion of the dose that becomes covalently bound to liver proteins in rats remains relatively constant only until a dose of 1.2 mmole/kg of bromobenzene is administered and is markedly increased when large doses are given (*29–31*). Moreover, centrilobular necrosis in liver does not occur unless doses larger than the threshold dose are given. As with acetaminophen, the dose threshold is due to the depletion of glutathione in liver (*29–31*). Accordingly, the proportion of the dose of bromobenzene excreted as the mercapturic acid decreases from about 70% to about 50% as the dose is increased from the nontoxic dose to a toxic dose (*30*). In addition, pulse-labeling experiments have revealed that the rate of covalent binding of radiolabeled bromobenzene is greater when glutathione concentrations in liver are low than when they are

high (*29*). Furthermore, *in vitro* experiments have shown that the rate of covalent binding is decreased by the addition of glutathione (*29*).

Pretreatment of rats with phenobarbital, which accelerates the metabolism of bromobenzene, increases the severity of the centrilobular necrosis and the amount of covalently bound metabolite (*28,29,31*). Although this finding indicates that the increase in covalent binding of the radiolabeled metabolite is caused by an increase in the rate of formation of bromobenzene 3,4-epoxide, the reason for the increase in covalent binding is subtle. Since all the bromobenzene is converted eventually to the epoxide metabolite, an increase in the rate of formation of the epoxide alone cannot cause a significant increase in the amount of covalent binding unless the rate of glutathione synthesis and the mobilization of other nucleophiles in the body is exceeded during the time that the epoxide is being formed in the body. Owing to the slow absorption of the toxic dose of bromobenzene from the intraperitoneal cavity (*30*), bromobenzene persists in the blood for as long as 6–10 hr in rats and hence considerable amounts of nucleophiles (presumably cysteine and glutathione) may be mobilized or synthesized. Thus, the steady-state concentration of the epoxide depends on the rates at which bromobenzene is absorbed and converted to the epoxide versus the rate of mobilization of nucleophiles (Fig. 3).

Another mechanism in addition to glutathione detoxification is operative. Since pretreatment of rats with phenobarbital increases the activity of the epoxide hydratase in liver endoplasmic reticulum (*12*), the amount of covalent binding should decrease after phenobarbital when the availability of glutathione for conjugation is not rate-limiting, because more of the epoxide should be detoxified by conversion to dihydrodiols (Fig. 3). In accord with this view, the pretreatment of rats with phenobarbital decreases the covalent binding of radiolabel when low, nontoxic doses of radiolabeled bromobenzene are administered, has little effect on it when intermediate doses are given and increases it when high doses are injected (*31,32*) (Table 4). Thus, the effects of pretreatments on the covalent binding of reactive metabolites may vary with the dose when the concentrations of cosubstrates for the conjugative reactions can become rate-limiting.

In contrast, the pretreatment of rats with 3-methylcholanthrene decreases both the covalent binding of radiolabeled bromobenzene to liver protein and the severity of liver necrosis (*30,33*).

Table 4. Effect of phenobarbital pretreatment on covalent binding to mouse liver proteins after different doses of ^{14}C-bromobenzene.[a]

Dose, mmole/kg	Covalent binding, nmole/mg protein	
	Untreated	Phenobarbital
0.13	0.074	0.027
1.15	0.79	0.99
4.85	0.44	9.83

[a] Data taken from Reid and Krishna (*31*).

Since the pretreatment does not alter the biological half-life of bromobenzene in rats (*30*) and actually increases the rate of bromobenzene metabolism by liver microsomes (*30*), the protective effect cannot be due to a decrease in the rate of bromobenzene elimination. Studies of the pattern of urinary metabolites in rats receiving a toxic dose of bromobenzene revealed that treatment with 3-methylcholanthrene resulted in a decrease in the mercapturic acid and 4-bromophenol and an increase in the bromocatechol, bromobenzene dihydrodiol and 2-bromophenol (*30*). Since 2-bromophenol cannot be formed from the nonenzymatic rearrangement of bromobenzene 3,4-epoxide, it seems likely that 3-methylcholanthrene induces the formation of a different epoxide, presumably bromobenzene 2,3-epoxide. Moreover, the increase in the bromocatechol and the bromobenzene dihydrodiol fractions at the expense of mercapturic acids in the urine of 3-methylcholanthrene-treated rats implies that the epoxide hydratase as well as the cytochrome P-450 enzyme was induced (*30*). Furthermore, *in vitro* studies revealed that in the absence of glutathione, the rate of covalent binding of radiolabeled bromobenzene by microsomes from 3-methylcholanthrene pretreated rats was slower that that by liver microsomes from untreated rats even though the rate of bromobenzene metabolism was faster (*34*). Increasing the formation of the bromobenzene 2,3-epoxide thus decreases the proportion of the dose of bromobenzene that is converted to the bromobenzene 3,4-epoxide, which may be the more reactive arylating intermediate. At the same time, increasing the activity of epoxide hydratase decreases the dependence of hepatocytes on glutathione in the inactivation of the epoxides and hence decreases its rate of utilization. When the rate of conjugation of glutathione is decreased, its concentration in hepatocytes can be maintained at high levels by its synthesis and by the mobilization of other nucleophiles, such as cysteine from body stores.

The net effect is thus a decrease in the covalent binding and hepatotoxicity of bromobenzene.

After the administration of radiolabeled bromobenzene, covalently bound radiolabel is found not only in the liver but also in a number of other tissues and blood plasma (*31*). The finding of covalently bound radiolabel in plasma raised the possibility that bromobenzene epoxide was sufficiently stable to leave the liver and be carried by the blood to other organs, although it still was possible that the covalently bound bromobenzene metabolites could have been released from damaged cells or associated with newly synthesized albumin during its passage through the lumen of the endoplasmic reticulum. On the other hand, the finding that bromobenzene metabolites became covalently bound during incubation *in vitro* with cell free tissue preparations, such as lung microsomes (*32*), also suggested that reactive metabolites of bromobenzene could be formed in extrahepatic tissues. Since pretreatment of mice or rats with phenobarbital increased the covalent binding of bromobenzene by liver microsomes but did not affect its rate of covalent binding by lung microsomes (*32*), it was possible to determine whether bromobenzene epoxide was sufficiently stable to enter the blood by studying the effects of phenobarbital on the covalent binding of radiolabeled bromobenzene in extrahepatic tissues *in vivo*. Such studies revealed that phenobarbital increased the covalent binding of bromobenzene to macromolecules in both lung and liver of mice receiving toxic doses of bromobenzene (*32*). It may, therefore, be concluded that the epoxide of bromobenzene can escape the liver after depletion of glutathione and can be carried to the lung where it becomes covalently bound. These studies thus demonstrated that the finding of an enzyme that catalyzes the formation of a reactive metabolite in an extrahepatic tissue does not necessarily mean that all of a reactive metabolite that becomes covalently bound in that tissue is formed there.

Furosemide

Recently, large doses of this drug were found to cause massive hepatic necrosis in mice (*35*). It is likely that the liver damage is probably caused by a reactive metabolite because both the necrosis and the covalent binding of radiolabeled furosemide is decreased by inhibitors of drug metabolism, such as piperonyl butoxide, cobaltous chloride, and α-naphthylisothiocyanate (*11,35*).

As with acetaminophen and bromobenzene, there is a dose threshold for the necrosis and the covalent binding of furosemide. No covalent binding, necrosis or alteration in the pattern of metabolism occurred until the dose exceeded 100 mg/kg. Unlike the dose threshold for bromobenzene or acetaminophen, however, the threshold for furosemide is not due to a depletion of glutathione in liver because toxic doses of furosemide do not appreciably decrease the glutathione concentration (*11,35*). Instead, the threshold is apparently caused by a change in the proportion of the dose that is eliminated unchanged as the dose is increased. At low doses of the drug, most of it is highly bound to plasma proteins and virtually all of it is eliminated unchanged (*11,35*). As the dose is increased, the plasma concentration is increased to a level that saturates the anionic binding sites on plasma proteins and the proportion of the dose that is available to the liver for metabolism is increased (Fig. 4) (*11,35*). There

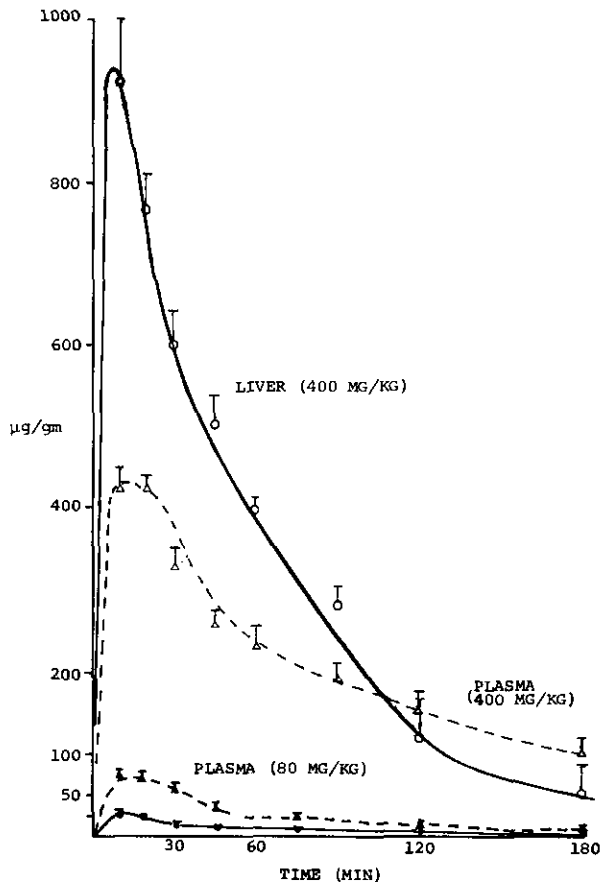

FIGURE 4. Change in tissue distribution of furosemide after toxic and nontoxic doses to mice: (——) liver; (– –) plasma.

are two plausible mechanisms which could account for these unusual effects. In one mechanism the transport system for the drug into bile may become saturated as the unbound concentration of the drug is markedly increased after doses that saturate the binding sites on plasma proteins. As a consequence more free furosemide is available to the liver for metabolism. In the other mechanism, the rate of elimination of the drug by the kidney is limited mainly by the blood flow rate through the kidney (even though the drug is highly bound to plasma proteins) while the rate of metabolism by the liver is limited mainly by the unbound concentration of the drug in plasma. According to this view, the saturation of the anionic sites on the plasma proteins that occurs after the administration of large doses of furosemide increases the unbound proportion of the drug in plasma and thus increases the rate of metabolism of the drug by the liver but does not appreciably affect its rate of elimination by the kidney. Both of these mechanisms, however, illustrate how physiological mechanisms of drug elimination can affect the pattern of drug elimination and thereby affect the kinetics of formation of chemically reactive metabolites.

Studies on the *in vitro* covalent binding of furosemide to liver microsomes have revealed that the furan moiety of furosemide is the part of the molecule that is activated by a cytochrome P-450 enzyme (Fig. 5). Since furosemide radiolabeled with tritium in the furan moiety was covalently bound to hepatic microsomes in the presence of NADPH and air to the same extent as furosemide radiolabeled with ^{35}S in its sulfonamide moiety, the bound metabolite must contain both parts of the furosemide molecule. When the metabolite protein conjugates isolated from liver were hydrolyzed under mild acidic conditions (pH 1.5), which splits furosemide into the methyl furan and the sulfamoyl anthranilic acid portions, the covalently bound tritum was retained by the protein, whereas the covalently bound ^{35}S was lost. Thus, the metabolic activation must have occurred on the furan ring, possibly through the formation of a furan epoxide (Fig. 5).

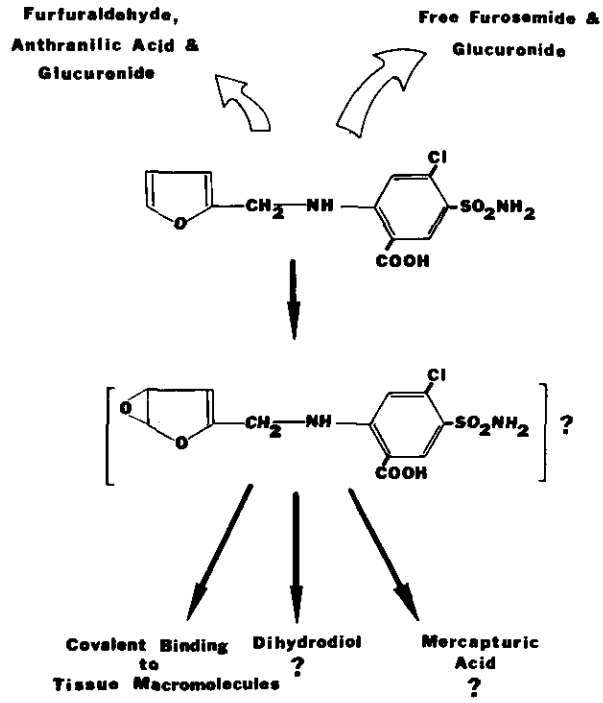

FIGURE 5. Proposed pathways of furosemide metabolism.

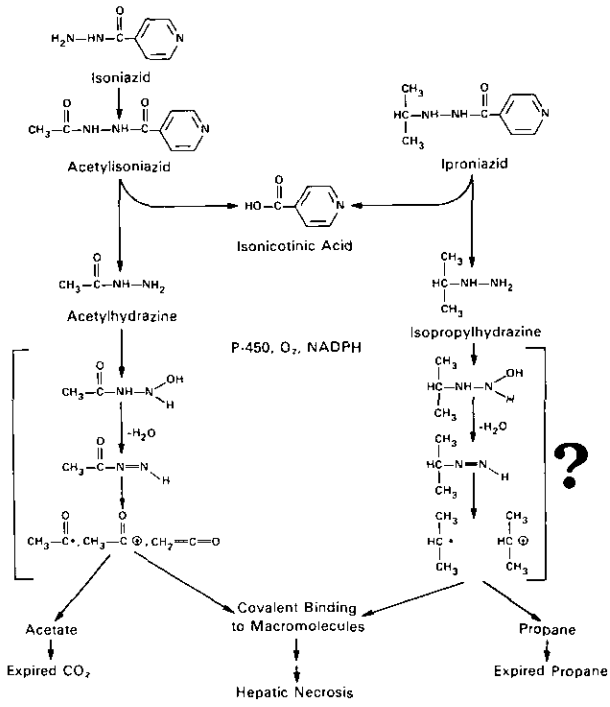

FIGURE 6. Proposed metabolic activation pathways for isoniazid, acetylisoniazid and isopropylisoniazid (iproniazid).

Isoniazid and Iproniazid

Isoniazid, used in the treatment of tuberculosis, causes a liver injury that resembles viral hepatitis (*36,37*). Recent evidence indicates that this toxicity may be mediated by a chemically reactive metabolite formed from acetylisoniazid, the major metabolite of isoniazid (Fig. 6). This

possibility was suggested by studies of isoniazid metabolism in which an unusually high proportion of patients with liver injury were found to be genetically fast acetylators of isoniazid (*36–39*).

Studies in rats revealed that acetylisoniazid in doses greater than 100 mg/kg caused rare, scattered, single cell necrosis in the liver, but after the animals were pretreated with phenobarbital these doses caused a marked hepatic necrosis (*9,39,40*). Moreover, the necrosis was prevented by treatment with cobaltous chloride, aminotriazole and piperonyl butoxide, suggesting that the necrosis was caused by a metabolite of acetylisoniazid.

By contrast, the parent compound, isoniazid, did not cause hepatic necrosis in either untreated or phenobarbital-pretreated rats, even after lethal doses. However, the proportion of the dose of isoniazid that is excreted as acetylisoniazid decreases markedly at doses greater than 100 mg/kg, suggesting that the acetylase becomes saturated after the administration of large doses of the drug. Consequently, when a large dose was divided into six 100 mg/kg doses and given hourly, the drug caused acute hepatic necrosis in phenobarbital-pretreated rats.

The administration of ^{14}C-acetyl labeled acetylisoniazid to rats resulted in considerable amounts of covalently bound radiolabel in the liver of rats (*9,39,40*). The proportion of the dose that became covalently bound was increased by prior treatment of the animals with phenobarbital and was decreased by treatment with cobaltous chloride or aminotriazole. However, no covalently bound radiolabel was found after the administration of acetylisoniazid labeled in the pyridine ring of the drug. Thus, the bound portion of the reactive metabolite originates from the acetyl moiety, formed endogenously in the body, rather than from isoniazid itself (Fig. 6). These findings illustrate the importance of studying the covalent binding of conjugates of foreign compounds before it is concluded that a given foreign compound does not form chemically reactive metabolites.

A comparison of the urinary metabolites of isoniazid and acetylisoniazid in rats revealed that most of the isonicotinic acid excreted into urine after the administration of isoniazid must be formed from acetylisoniazid because the relative amounts of acetylisoniazid and isonicotinic acid excreted into urine were nearly identical after the administration of either isoniazid or acetylisoniazid (*11,39,40*). Thus, it seems likely that the liver necrosis in rats is mediated by acetylhydrazine or one of its metabolites. Indeed, in phenobarbital-pretreated rats, acetylhydrazine caused midzonal and centrilobular necrosis in doses as low as 15 mg/kg (*9,39,40*). Since the necrosis caused by acetylhydrazine could still be inhibited by cobaltous chloride, aminotriazole and piperonyl butoxide, however, the acetylhydrazine must be further activated in the body. In fact, the covalent binding of radiolabeled acetylhydrazine by rat liver microsomes is catalyzed by cytochrome P-450 in liver microsomes (*39-41*).

The liver toxicity caused by iproniazid probably occurs through a similar sequence of events (Fig. 6). Like acetylisoniazid, iproniazid undergoes hydrolysis to isonicotinic acid and a hydrazine derivative (*39–41*). The isopropylhydrazine formed in the body, however, may be more toxic than acetylhydrazine because the K_m value for covalent binding of isopropylhydrazine by rat liver microsomes is an order of magnitude lower than that for acetylhydrazine (*41*).

Summary

In this paper we have discussed an integrated approach for relating the formation of chemically reactive metabolites with the incidence and severity of toxicities caused by foreign compounds. The approach emphasizes the need to correlate the findings of several different kinds of studies including the measurement of the biological half-life of the foreign compound, the quantitative isolation of its urinary metabolites and the determination of the amount of covalent binding of reactive metabolites both *in vivo* and *in vitro*. It emphasizes the importance of determining how various treatments affect these parameters.

Although such data by themselves can neither predict the toxicity of an unknown compount without morphometric studies nor elucidate the mechanism by which cell death occurs, they nevertheless have served an essential role in showing that many drugs and other foreign compounds cause liver damage through the formation of chemically reactive metabolites. The concepts discussed in this paper have been especially useful in reconciling seemingly conflicting data obtained in the different kinds of data. Indeed without these concepts, the mechanism by which phenobarbital evokes opposite effects on acetaminophen toxicity in mice and hamsters (*17,24*) would have been virtually impossible to uncover. The concepts also predict possible pitfalls in attempting to relate species differences in drug toxicity with species differences in the pattern of

urinary metabolites and in the biological half-lives of foreign compounds. They point out that studies of covalent binding are useful in identifying reactive metabolites of foreign compounds and the way in which their formation may be decreased. Such studies are also useful in identifying whether there are dose thresholds in the toxicity of a given foreign compound and the reasons for such dose thresholds. It seems likely that studies of covalent binding will also be useful in determining whether alterations in the incidence and severity of various toxicities are due to differences in the metabolism of the foreign compound or to changes in the events that follow the formation of the reactive metabolite. Because of these complexities, the value of examining the covalent binding of chemically reactive metabolites *in vivo* simultaneously with the determination of tissue injury and the pattern of urinary metabolites is apparent when elucidating mechanisms of chemically induced tissue lesions.

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