BIOCHEMICAL MECHANISMS OF DRUG TOXICITY[1]

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INTRODUCTION

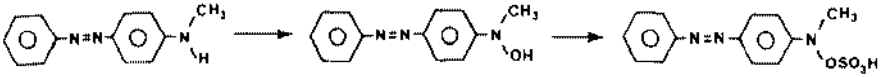
It has long been known that many drugs can be converted in the body to various metabolites that evoke therapeutic and toxicologic responses. In most instances these metabolites are chemically inert and bring about their effects by combining reversibly with action sites in tissues. In some instances, however, drugs and other foreign compounds can be converted in the body to chemically reactive metabolites which either uncouple integrated biochemical processes in cells or combine covalently with various tissue macromolecules, such as DNA, RNA, protein, and glycogen. During the past several years it has become increasingly evident that chemically reactive metabolites mediate many different kinds of serious toxicity, including carcinogenesis, mutagenesis, cellular necrosis, hypersensitivity reactions, methemoglobinemia, hemolytic anemia, blood dyscrasias, and fetotoxicities. This review is devoted mainly to the mechanisms by which various kinds of toxicities are mediated by chemically reactive metabolites of drugs and the factors that affect the severity of the toxicities.

Since the pioneering work of the Millers in Wisconsin and of Magee and coworkers in England, it has become increasingly evident that most if not all chemical carcinogens bring about their effects by combining covalently with DNA and other tissue macromolecules or by being transformed to chemically reactive metabolites that in turn combine covalently with tissue macromolecules (1–4).

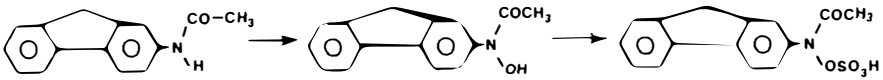
The many studies on the mechanism of formation of carcinogenic metabolites in the body have revealed that chemically inert substances can be converted to chemically reactive metabolites by a variety of different reactions (Figure 1). For example,

[1]The following abbreviations are used: SKF 525-A, β-diethylaminoethyl diphenylpropylacetate; ANIT, α-naphthylisothiocyanate; DDT, 1,1,1-trichloro-2,2-bis (*p*-chlorophenylethane); GSH, glutathione; CCl_4, carbon tetrachloride; $CHCl_3$, chloroform.

secondary amines, such as N-methyl-4-aminoazobenzene, primary amines including β-naphthylamine and aminobiphenyl, and acetylated primary amines including 2-acetylaminofluorene are N-hydroxylated by either cytochrome P-450 enzymes or amine N-oxidase. In some instances the metabolites are further activated by being converted to N-O-sulfate esters (1, 2, 4). Dialkylnitrosamines are N-demethylated by cytochrome P-450 enzymes to monoalkylnitrosamines, which in turn spontaneously rearrange to unknown active metabolites, possibly alkyl carbonium ions (3).

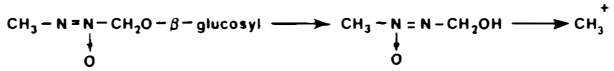


N-Methyl-4-aminoazobenzene

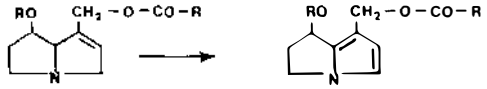


2-Acetylaminofluorene

Dimethylnitrosamine

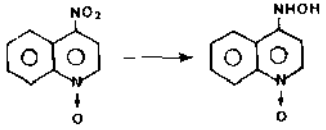


Cycasin



Pyrrolizidine Alkaloids

Polycyclic hydrocarbons



4-Nitroquinoline N-oxide

Figure 1 Biochemical mechanisms for the formation of chemically reactive metabolites.

Cycasin is hydrolyzed in the intestine by bacterial β-glucosidase to methylazoxymethanol which acts as a methylating agent (5). Urethan may undergo an N-hydroxylation before it is converted to an ethylating agent (6). Pyrrolizidine alkaloids are thought to be dehydrogenated to chemically reactive pyrrole derivatives (7, 8). Polycyclic hydrocarbons undergo epoxidation by cytochrome P-450 enzymes to form potent arylating agents (9). Nitroaryl compounds, such as 4-nitroquinoline N-oxide, may act either as arylating agents per se or be converted to chemically reactive hydroxylamine derivatives (10). Similar reactions have also been implicated in the formation of chemically reactive metabolites that effect other kinds of toxicity rather than carcinogenesis or mutagenesis. Presumably these metabolites bring about their effects by combining with macromolecules other than DNA or RNA.

TOXICITIES BY THERAPEUTIC AGENTS

The possibility that therapeutic agents as well as environmental agents cause various kinds of pathological lesions in addition to maligant tumors through the formation of active metabolites occurred to us several years ago (11). The evaluation of this possibility, however, has been difficult because potential therapeutic agents that cause pathological lesions reproducibly in animals are usually eliminated from consideration by carefully performed animal toxicity tests. For the rational development of nontoxic therapeutic agents, therefore, the factors that control the severity of the lesions need to be better understood. In this regard there are two sets of factors to the problem: in one set are those factors that control the formation and reactivity of the active metabolite and in the other set are those factors that affect the severity of the lesion after the active metabolite reacts with its action sites. Although for most drug-induced toxicities little is known of the latter set of factors, considerable progress has been made in elucidating those factors that control the formation and fate of active metabolites.

For many years it has been realized that the effects of an active metabolite that acts by combining reversibly with action sites can be frequently related to its plasma level. But when the response is tissue damage caused by the covalent binding of chemically reactive metabolites to tissue macromolecules, it would not be logical to expect a relationship between the plasma level of the active metabolite and the severity of the lesions. Indeed, with highly reactive metabolites, little or none would reach the plasma. Nevertheless, for any particular compound there should be a relationship between the severity of the lesion and the amount of covalently bound active metabolite.

Hepatic Necrosis

Many of the ways by which the severity of drug-induced tissue damage can be affected by changes in either the formation or fate of chemically reactive metabolites can be illustrated by studies on the mechanism of liver necrosis induced by halobenzenes. Although these compounds are chemically inert, they can be converted by a cytochrome P-450 enzyme in liver microsomes to their epoxides, some of which become covalently bound to macromolecules. Recently it was found that the magni-

tude of the covalent binding of radiolabeled metabolites caused by these compounds paralleled their toxic effects; at doses of 1.0 mmol/kg administered to rats, chlorobenzene, bromobenzene, iodobenzene, and *o*-dichlorobenzene lead to liver necrosis and considerable covalent binding of reactive metabolites to liver protein, whereas fluorobenzene and *p*-dichlorobenzene do not cause liver necrosis and are not appreciably bound (12–14). Moreover, studies with ^{14}C-bromobenzene revealed that the covalent binding of its chemically reactive metabolite occurs predominately in the centrilobular necrotic areas of liver. Pretreatment of rats with phenobarbital, a compound that stimulates the activity of the cytochrome P-450 drug-metabolizing enzymes in liver, also increases the rate of disappearance of bromobenzene from the body, the covalent binding of radiolabeled bromobenzene in liver, and the severity of liver necrosis (13, 15, 16). In contrast, SKF 525-A (β-diethylaminoethyl diphenylpropylacetate) and piperonyl butoxide (27), compounds that decrease the activity of drug-metabolizing enzymes in liver, also decrease the rate of disappearance of bromobenzene, the covalent binding of radiolabeled bromobenzene, and the severity of the liver necrosis (13, 15–18). Thus, the necrosis is caused by the covalent binding of a metabolite and not by bromobenzene itself, and the severity of the necrosis apparently depends on the rate at which the active metabolite is formed.

The nature of the active intermediate was confirmed in vitro by studies using liver microsomes, labeled substrate and glutathione (GSH) (12, 18, 19). The results showed that bromobenzene in the presence of TPNH and O_2 is activated by microsomes to a substance that reacts covalently with GSH, thereby trapping the active intermediate. The amount of this complex is increased in microsomes from rats pretreated with phenobarbital. In addition, carbon monoxide, which lowers the activity of the cytochrome P-450 enzymes, decreases the formation of the complex. Thus, the reactive metabolite is formed by a cytochrome P-450 enzyme.

Dose response studies with bromobenzene in rats have revealed that the proportion of the dose that becomes covalently bound to liver microsomes remains low until a critical dose between 1.20 and 2.15 mmol/kg is used (13, 14). Above this critical dose the proportion of the dose that becomes covalently bound is nearly doubled, and the necrosis is manifested. Moreover, the effects of phenobarbital pretreatment depend on the dose of bromobenzene used; with subtoxic doses of bromobenzene, pretreatment with phenobarbital decreased the covalent binding in rats, whereas with high doses of the toxicant, the pretreatment increased the covalent binding.

The reason for the threshold dose of bromobenzene and the dichotomous effects of phenobarbital was clarified by studies on the pathways of bromobenzene metabolism. The major route of metabolism of bromobenzene is through the formation of 3,4-bromobenzene epoxide (9, 19, 20). Liver cells, however, have a number of ways of converting the epoxide to chemically inert metabolites. The epoxide can rearrange to form *p*-bromophenol (9, 19). An enzyme in the soluble fraction of liver can catalyze a reaction between the epoxide and GSH to form a GSH conjugate (12) that ultimately is excreted in urine as a mercapturic acid (21, 22). Another enzyme present in the endoplasmic reticulum of liver can catalyze the addition of water to the epoxide to form a dihydrodiol which in turn can be oxidized to a catechol (20). The steady-state concentration of the epoxide in liver thus depends on the rate of

formation of the epoxide on the one hand and the rates at which the epoxide is converted to the phenol, the GSH conjugate, and the dihydrodiol on the other hand. When the concentration of the epoxide is high, it presumably would react with macromolecules in liver and thus lead to liver necrosis.

As bromobenzene is converted to its GSH conjugate, however, the GSH levels are decreased until the rate of formation of the conjugate is limited by the rate of GSH synthesis. Covalent binding and the severity of the necrosis would be expected to increase after the GSH stores in the liver had been depleted. This view was confirmed (19, 23) by taking advantage of the fact that large doses of bromobenzene administered intraperitoneally are slowly absorbed, and thus the liver levels of the toxicant are maintained for several hr (16), whereas, small doses of bromobenzene administered intravenously are rapidly metabolized with a half-life of about 9 min (16). Thus by administering a large dose of unlabeled bromobenzene intraperitoneally and then injecting radiolabeled bromobenzene intravenously at various times thereafter the magnitude of the covalent binding of the radiolabeled metabolites could be determined while the GSH levels were decreasing, were at their nadir levels, and were returning to normal. Little covalent binding of the radiolabeled bromobenzene occurred when GSH levels were high, but considerable binding occurred when they were low (19, 23). Thus, the degree of covalent binding of bromobenzene and the severity of necrosis depends on the liver levels of GSH, which in turn depend on the relative rates at which GSH is being synthesized and being consumed in the formation of the GSH conjugate.

Pretreatment of animals with phenobarbital increases the rate of bromobenzene epoxide formation and thus hastens the depletion of liver GSH. In addition, it may also increase the activity of GSH transferase as well as the epoxide hydrase in rats (9, 19), because it decreases the covalent binding of subtoxic doses of bromobenzene in this species (14). At toxic doses, however, pretreatment with phenobarbital increases both the amount of covalent binding of bromobenzene metabolites and the severity of the bromobenzene-induced liver necrosis (13) presumably because the increase in rate of bromobenzene epoxide formation exceeds the increase in hydrase activity and the rate of GSH conjugate formation is limited by GSH synthesis (19). By contrast, SKF 525-A inhibits the formation of bromobenzene epoxide and thus slows the depletion of liver GSH to such an extent that the liver levels of GSH are never severely depleted. As a consequence, the covalent binding (13) is markedly decreased and the toxicity almost completely prevented.

On the other hand, pretreatment of rats with 3-methylcholanthrene decreases the severity of bromobenzene necrosis (16, 116) by changing the relative proportions of the kinds of epoxide that are formed; very little 2,3-bromobenzene epoxide is formed in untreated rats, whereas considerable amounts of this epoxide is formed in 3-methylcholanthrene pretreated rats (16, 18). In addition, the protection by 3-methylcholanthrene may be due in part to an induction of epoxide hydrase (9, 19).

The concept that the pattern of metabolism can change with the dose also accounts for the finding that drugs may not be toxic unless a certain threshold dose is exceeded. For example, acetaminophen is among the safest of all minor analgesics when taken in normal therapeutic doses. But large overdoses of acetaminophen can produce fatal hepatic necrosis in man (24), rats (25, 26), and mice (26).

In unpretreated mice, acetaminophen does not cause centrilobular necrosis in the liver unless the dose is greater than about 300 mg/kg (26). Pretreatment of the mice with phenobarbital, however, markedly increases the severity of the toxicity (26), whereas pretreatment of mice with piperonyl butoxide, cobaltous chloride or α-naphthylisothiocyanate (ANIT), which decrease the activities of the microsomal cytochrome P-450 enzymes (27–29), decrease the severity of the necrosis (26, 30).

As with the halobenzene derivatives, the severity of the necrosis parallels the magnitude of the covalent binding of radiolabeled acetaminophen to liver proteins and the decrease in liver GSH levels (31, 32, 34). For example, little covalent binding and no liver necrosis occurs at doses of acetaminophen that deplete liver GSH less than 85%. But considerable binding and liver necrosis occurs with doses that deplete liver GSH more than 85%. Moreover, pretreatment of mice with phenobarbital increases the covalent binding, the rate at which acetaminophen depletes the liver GSH and the severity of the necrosis, whereas pretreatment with cobaltous chloride, piperonyl butoxide or ANIT decreases them (30, 31, 34).

These findings suggest that GSH in liver prevents the necrosis and the covalent binding by reacting with an active metabolite of acetaminophen, presumably N-hydroxy-N-acetyl-*p*-hydroxyaniline (32, 32a). In accord with this view, the prior administration of diethyl maleate, which depletes liver GSH (33) without causing necrosis, increases the covalent binding of acetaminophen metabolites to liver protein and the severity of acetaminophen-induced liver necrosis (34). But the prior administration of cysteine, which leads to the synthesis of GSH, or of cysteamine or dimercaprol, which presumably react chemically with the reactive metabolite, decreases both the covalent binding of the acetaminophen metabolite and the severity of liver necrosis (34, 35). Thus, a fundamental role of GSH may be to protect essential thiol and other nucleophilic groups in tissue macromolecules from electrophilic reactants formed in animals.

The significance of GSH in protecting man from acetaminophen and other drug-induced hepatic damage is still uncertain, but it probably is responsible for the remarkable safety of the drug after usual therapeutic doses. For example, the toxic metabolite of acetaminophen can be shown to combine preferentially with GSH in animals to form a nontoxic conjugate that is ultimately excreted as a mercapturic acid (34–37). As the dose of acetaminophen is increased to levels that deplete hepatic GSH and cause significant covalent binding and liver necrosis, the proportion of the dose excreted as a mercapturic acid decreases (38). The measurement of acetaminophenmercapturic acid in urine has been used to estimate the formation of the toxic metabolite of acetaminophen in humans. In 12 patients the amount of mercapturate formed was always about 2% of the administered dose over a dose range from 600 to 1800 mg, demonstrating that the availability of GSH was never limiting after these therapeutic doses (35, 38). In addition, phenobarbital pretreatment of these patients for 5 days increased the amount of acetaminophen excreted as a mercapturate from 2% to about 4.5% of the therapeutic doses, suggesting an increased formation of the toxic metabolite after phenobarbital induction but again without exceeding the availability of GSH at these doses. These data suggest that the hepatic toxicity after acetaminophen overdosage might be increased in humans receiving

inducers such as phenobarbital. In fact, a retrospective study of patients suffering from acetaminophen-induced hepatic necrosis has confirmed this view (39).

Because acetaminophen-induced hepatic damage and covalent binding in animals are prevented, but not reversed, by a variety of exogenously administered nucleophiles, such as cysteamine and dimercaprol (34, 35), these substances may provide a possible rationale for treatment of overdosed patients seen early after poisoning.

The formation of the chemically reactive metabolite of acetaminophen represents a relatively minor pathway of the metabolism of the drug in mice and hamsters as well as in man. Indeed, most of the drug in animals is excreted as its glucuronide and sulfate conjugates (40), which are not formed by cytochrome P-450 enzymes. As a result, pretreatment of mice with phenobarbital or cobaltous chloride do not alter the biological half-life of the drug even though these treatments markedly affect its toxicity (26). Moreover, although these treatments cause statistically significant changes in the pattern of urinary metabolites (38), the changes may not appear impressive and could have been easily overlooked. These findings illustrate the problem of finding an animal species which mimics humans in the metabolism and toxicity of drugs when the toxicity is mediated by metabolites formed along minor pathways.

Another commonly used drug, furosemide, is also safe at the usual therapeutic doses but at high doses produces a dose-dependent hepatic necrosis in male mice (35, 41). The necrosis is restricted to midzonal and centrilobular hepatocytes after doses of 150 mg/kg, ip. Larger doses (400 mg/kg) produce a massive necrosis in which confluent zones of anuclear and eosinophilic cells bridge adjacent hepatic lobules. Inhibitors of cytochrome P-450 enzymes, such as piperonyl butoxide, cobaltous chloride and ANIT, prevent furosemide-induced hepatic necrosis, which suggests that furosemide-induced hepatotoxicity may also be mediated by toxic metabolites (35, 41).

As with the toxic halobenzenes and acetaminophen, the severity of necrosis after these pretreatments parallels the amounts of furosemide metabolites that are covalently bound to liver macromolecules (42). In addition, dose response curves have revealed that little covalent binding occurs below a critical dose of 150 mg/kg. But furosemide, unlike the halobenzenes and acetaminophen, does not deplete the liver of GSH. Thus, the reason for the threshold is unclear, but preliminary evidence suggests that it may be due to saturation by the drug of the reversible binding sites of plasma proteins either alone or in combination with the saturation of the active secretory systems in the kidney (38).

In the past CCl_4 has been used as an anthelmintic, and $CHCl_3$ has been used as an anesthetic gas. But the well-known toxicities caused by these substances soon led to their disuse in man. Indeed, the mechanism of CCl_4-induced liver necrosis has become the subject of numerous studies and reviews (43–46). Only during the past few years, however, has the mechanism by which CCl_4 and $CHCl_3$ been clarified.

A number of studies have provided indirect evidence that the toxic actions of CCl_4 and $CHCl_3$ are mediated through an active metabolite. For example, the lethal effects of CCl_4 were increased by prior administration of phenobarbital (47), 1,1,1,-trichloro-2,2-bis (*p*-chlorophenylethane) (DDT) (47), or isopropyl alcohol (48).

Moreover, the toxic effects were diminished by the administration of dibenamine (49) or cobaltous chloride (50) or by feeding the animals protein-deficient diets (47). Similarly the liver necrosis caused by chloroform is also increased by pretreatment of rats with phenobarbital (43). Moreover, the finding that radiolabeled CCl_4 became irreversibly bound to proteins (51–53) and to lipids (52, 54) led to the concept that the active metabolite of CCl_4 combines covalently with proteins and lipids and thereby causes centrilobular necrosis, presumably by promoting lipid peroxidation (43–46). In accord with this view, pretreatment of rats with dibenamine prevents the toxic effects of CCl_4 and decreases the covalent binding of CCl_4 metabolites to lipids presumably by impairing the liver enzyme system that catalyzes the formation of the reactive metabolite (49). On the other hand, pretreatment of rats with phenobarbital or isopropyl alcohol increases not only the toxic effects but also the covalent binding of CCl_4 in vivo (23, 55, 56).

Many years ago, Butler raised the possibility that CCl_4 was homolytically split to form the free radicals, $CCl_3\bullet$ and $Cl\bullet$, in the body (57). It now appears, however, that the formation of the chemically reactive metabolite occurs by reductive cleavage of CCl_4 to $CCl_3\bullet$ and chloride ion. Covalent binding of CCl_4 metabolites occurs in incubation mixtures consisting of liver microsomes, NADPH and $^{14}CCl_4$ (58–60). The binding is inhibited by CO (58–60) and by an antibody against liver microsomal NADPH cytochrome *c* reductase (61); thus the enzyme that catalyzes the formation of the free radical is probably a cytochrome P-450 enzyme system. Moreover, covalent binding of CCl_4 occurs to a greater extent under anaerobic conditions than in air (60, 61), indicating that the reaction is reductive rather than oxidative. On the other hand, the covalent binding of $^{14}CHCl_3$, which is also mediated by a cytochrome P-450 system in liver microsomes, does not occur under anaerobic conditions (62), indicating that the activation is oxidative rather than reductive. It is also noteworthy that the covalent binding of CCl_4 by liver microsomal preparations in the presence of NADPH is also decreased by pretreating rats with dibenamine (63) and increased by pretreating them with phenobarbital (61) or isopropyl alcohol (64).

Although GSH does not appreciably decrease the in vitro covalent binding of CCl_4 metabolite to rabbit liver microsomes (60), it markedly inhibits the covalent binding of CCl_4 to rat liver microsomes (58, 59). Thus it seemed possible that GSH may also tend to protect the liver against the toxic effects of CCl_4, even though the liver levels of GSH are not depleted after the administration of CCl_4 to rats. In accord with this view, prior administration of diethyl maleate, which depletes liver GSH (33) without causing toxicity, increases both the toxicity of CCl_4 and the covalent binding of CCl_4 metabolites in vivo (23, 56). The mechanism by which GSH diminishes covalent binding, however, remains to be clarified.

In addition to causing centrilobular necrosis and fatty infiltration of liver, CCl_4 also causes the destruction of liver microsomal cytochrome P-450. As a consequence, the toxic effects of CCl_4 may be self-limiting. Indeed, the administration of sublethal doses of CCl_4 to animals protects them from the lethal effects of high doses of CCl_4 (65). The mechanisms by which CCl_4 causes the destruction are not yet clear. Studies in vitro have shown that the CCl_4-induced destruction of cytochrome

P-450 may be mediated by lipid peroxidation, because EDTA added to liver microsomal systems blocks both the NADPH-dependent lipid peroxidation and the cytochrome P-450 destruction caused by CCl_4 (66). However, the finding that the administration of the antioxidants, α-tocopherol or diphenylphenylenediamine, does not affect the CCl_4-induced destruction of cytochrome P-450 seemed to contradict this view (67). But this interpretation of the in vivo results may have been incorrect because it was recently found that the antioxidants have little effect on lipid peroxidation induced by CCl_4 in vivo as measured by diene conjugation of liver microsomal lipids (68).

A mechanism similar to that of CCl_4 may play an etiologic role in the clinical hepatitis seen after halothane anesthesia, because microsomes isolated from the livers of rats pretreated with phenobarbital followed by halothane had elevated lipid diene conjugates and decreased cytochrome P-450 content (69). In spite of these interesting preliminary results, however, final conclusions must await more definitive experiments.

Liver damage caused by other kinds of foreign compounds may also be mediated by chemically reactive metabolites. For example, there is considerable evidence that the hepatic necrosis caused by pyrrolizidine alkaloids is mediated by a chemically reactive intermediate that becomes covalently bound to liver macromolecules (70–72). There is also evidence that the toxic effects of sesquiterpenes (73), ANIT (29), and the toxins in amanita toadstools (74, 75) are also mediated through active metabolites, because either stimulators of drug metabolism increase their toxic effects or inhibitors prevent the toxicities.

Lung Necrosis

Although a number of compounds are known to cause lung toxicities, including carcinoma and necrosis, the mechanisms by which these toxicities occur is poorly understood. However, a recent study shows that bromobenzene causes lung necrosis apparently by being covalently bound to lung macromolecules (76), which suggests the possibility that at least some substances, including drugs, might evoke their toxic effects by being converted to chemically reactive metabolites. In mice, bromobenzene causes necrosis of bronchiolar and bronchial cells but does not cause any specific alterations in alveolar morphology. In rats, bromobenzene caused similar but less severe pathological changes. After the administration of ^{14}C-bromobenzene, autoradiograms revealed a preferential accumulation of covalently bound bromobenzene metabolite in the bronchi and bronchioles in both rats and mice. In vitro studies revealed that lung microsomes contain a cytochrome P-450 enzyme system that converts bromobenzene to a reactive intermediate that becomes covalently bound to lung microsomal proteins and that the system is more active in mice than in rats. But pretreatment with phenobarbital does not increase the activity of the enzyme system in lung of either species. With subtoxic doses of bromobenzene, pretreatment with phenobarbital did not affect the in vivo covalent binding of ^{14}C-bromobenzene metabolites to liver and lung protein in mice and decreased it in rats. But with toxic doses, pretreatment of mice with phenobarbital increased the covalent binding in both liver and lung. The finding that phenobarbital increases the

covalent binding of bromobenzene in the lung without inducing the lung enzyme suggests that the chemically reactive metabolite of bromobenzene can escape the liver after depletion of GSH and can be carried to the lung where it becomes covalently bound to proteins in the lung. In accord with this view, phenobarbital pretreatment increases the small amounts of ^{14}C-bromobenzene covalently bound to heart muscle in vivo, a tissue that does not metabolize bromobenzene.

Not all chemically induced toxicities in the lung are caused by covalent binding of chemically reactive metabolites, however. For example, paraquat causes hemorrhage, edema, and fibrosis in rat lung (77), but does not become covalently bound to lung macromolecules (78). Indeed, the mechanism by which paraquat evokes these pathological changes is not clear. The realization that paraquat, also called methyl viologen, is a well-known redox dye, raised the possibility that paraquat might act by promoting the formation of hydrogen peroxide and thereby cause tissue damage (79). Indeed in vitro experiments have shown that paraquat at high concentrations does stimulate NADPH oxidation and hydrogen peroxide formation (78, 79). But these reactions are insignificant at the concentrations of paraquat found in vivo (78).

Renal Toxicity

Low doses of chloroform (2.5 mmol/kg) cause necrosis of the epithelial cells of the proximal convoluted tubules of kidney in male C-57 Black mice but not in females. In accord with the view that the toxicity is mediated by covalent binding of chloroform metabolites, the in vivo covalent binding of ^{14}C-$CHCl_3$ was only about one-tenth as much in kidneys of females as it was in those of males (80). Moreover, pretreatment of male mice with either phenobarbital or piperonyl butoxide decreased both the covalent binding of the reactive chloroform metabolite and the toxicity.

Kidney toxicities, however, are not always mediated by covalent binding of metabolites. For example, the polyuric, vasopressin-resistant renal insufficiency associated with the administration of methoxyflurane
related in man and can be correlated with high levels of serum inorganic fluoride, a metabolite of methoxyflurane (81). The lesion can be reproduced in rats either by administration of methoxyflurane or a comparable dose of the fluoride ion. As might be expected, sensitive rat strains metabolize methoxyfluorane to a greater extent than do insensitive strains (82). Moreover, pretreatment of the animals with phenobarbital increased the severity of the toxicity, whereas the administration of SKF 525-A decreased it (83).

Testes Toxicity

Although spironolactone induces cytochrome P-450 enzymes in liver (84–86), it causes a selective destruction of testicular cytochrome P-450 and thereby decreases the synthesis of testosterone (87). Since NADPH is required for the destruction of testicular cytochrome P-450 by spironolactone in vitro, the effect probably is mediated by an active metabolite (88). But neither the identity of the active metabolite nor its mechanism of action is known. The impairment in testicular cytochrome

P-450 is not due to cellular necrosis and is fully reversible within 5 days after withdrawal of the drug (87). Experiments in vivo in male dogs demonstrate that spironolactone not only decreases testicular cytochrome P-450 but also decreases the release of both testosterone and estradiol into the plasma (89). As in animals, spironolactone also caused decreases in the plasma levels of testosterone in patients (90, 90a). But whether the decrease is due mainly to decreased synthesis in testis or increased catabolism in liver is not known. It is also not known whether these effects of spironolactone on the metabolism of sex steroids account for the gynecomastia and decreased libido observed in humans receiving the drug.

Nitrofurazone, a compound used as a topical antibacterial agent, is known to cause mammary tumors in rats (91). Moreover, it also causes a decrease in testicular cytochrome P-450, and impairs testosterone synthesis in mice (92). In addition, it causes aspermatogenesis (92, 93) as manifested by a decrease in spermatozoa, increased vacuolization of the spermatocytes and an increase in polynucleated spermatotides. Aspermatogenesis also occurs with furadroxyl, an analog of nitrofurazone (94). Because nitrofurazone can be reduced to its hydroxylamine derivative by xanthine oxidase (95) and aldehyde oxidase (96) as well as by liver microsomal NADPH cytochrome *c* reductase (97), it is not surprising that it becomes covalently bound to proteins of various tissues in vivo including liver, kidney, and either testis or mammary glands (92). Strangely, nitrofurazone causes a decrease of GSH in liver but not in testes even though the toxicity occurs in testes but not in liver. The possible relationship between the toxic effects of the drug and covalent binding thus remains obscure.

In rats, testicular damage is also caused by high doses of the carcinogen, 7,12-dimenthylbenzanthracene (98). But again the mechanism by which the toxicity occurs is unknown.

Bone Marrow Aplasia

Studies on the toxic effects of chloramphenicol have been hampered by the inability of the drug to produce aplastic anemia in laboratory animals. Nevertheless, it may be important that chloramphenicol in rats becomes covalently bound to both bone marrow (100 pmoles/mg) and to liver (450 pmoles/mg) and that phenobarbital pretreatment increases the covalent binding in both tissues by about 2–3-fold (99).

Most studies on bone marrow aplasia have been carried out with model compounds. For example, it is well known that 7,12-dimethylbenzanthracene causes leukopenia, thrombocytopenia, and bone marrow aplasia (98). Recent studies have shown that the severity of the syndrome produced by 7,12-dimethylbenzanthracene could be reduced by inhibitors of cytochrome P-450, SKF 525-A, or 7,8-benzoflavone (100). Because 7,12-dimethylbenzanthracene is not metabolized by bone marrow preparations of rats, it seems likely that a metabolite formed in the liver mediates the toxicity.

Repeated injections of benzene also produce bone marrow aplasia in rats. The lesion is prevented when the metabolism of benzene is altered by a variety of pretreatments (SKF 525-A, piperonyl butoxide, phenobarbital) (101). Although the finding that phenobarbital pretreatment protects against benzene-induced bone mar-

row damage has led investigators to propose that benzene itself is the toxic agent (102), this seems unlikely because pretreatments such as cobaltous chloride and aminotriazole, which inhibit the synthesis of cytochrome P-450 in rats, block the metabolism of benzene and markedly reduce the bone marrow damage (103). Thus, it seems likely the bone marrow aplasia caused by benzene is mediated by an unknown active metabolite.

Methemoglobinemia and Hemolytic Anemia

It has been well established that aromatic amines including aniline cause methemoglobinemia by being converted to phenylhydroxylamines which on oxidation to their nitroso derivatives and subsequent reduction back to the phenylhydroxylamines promote the formation of methemoglobin (104, 105). In addition, certain drugs including aniline derivatives also cause hemolysis, especially in patients having a genetic deficiency in erythrocyte glucose-6-phosphate dehydrogenase (106). Because both kinds of toxicity can be caused by various aniline derivatives, it has not been clear whether they are mediated by the same or different metabolites of any given drug. In order to evaluate the severity of hemolysis, ^{51}Cr-labeled rat erythrocytes injected intravenously into rats have been used to measure the effects of drugs on the turnover rate of erythrocytes (35, 107, 108). After the intraperitoneal administration of aromatic amines, such as aniline, *p*-phenetidine and *p*-chloroaniline the rate at which ^{51}Cr declined in blood was greatly increased, confirming that these drugs induced hemolysis in rats. These drugs when added to incubation mixtures had no effect on erythrocyte survival, and the pretreatment of rats with CCl_4, which decreased the metabolism of the drugs prevented the hemolysis by the aromatic amines. Thus, the hemolysis is probably caused by metabolites rather than by the parent amines.

The corresponding N-acetylated derivatives, namely acetanilide, phenacetin, and *p*-chloroacetanilide, also caused hemolysis in this sytem, although to a lesser degree than did the free amines. The hemolysis produced by the acetylated amines was prevented by pretreatment of the rats with CCl_4 and by pretreatment with bis-*p*-nitrophenyl phosphate, which inhibits deacetylation (109). In contrast, the administration of the deacetylase inhibitor did not prevent the hemolysis induced by the free amines. These results suggest that these analgesic drugs require metabolic transformation in two steps in order to cause hemolysis, presumably deacetylation and N-hydroxylation (35, 107, 108).

Because methemoglobinemia caused in rats by phenacetin or acetanilide also is prevented by pretreatment with piperonyl butoxide and by bis-*p*-nitrophenyl phosphate, it might seem possible that the same active intermediate causes both hemolysis and methemoglobinemia. However, phenobarbital pretreatment of rats increases the methemoglobinemia but decreases the hemolysis caused by acetanilide and aniline, whereas pretreatment with an inhibitor of metabolism, piperonyl butoxide, brings about exactly the opposite effects (35, 107, 108). Although the identity of the metabolite that induces hemolysis remains obscure, the results clearly demonstrate that methemoglobinemia and drug-induced hemolysis are mediated by different active metabolites and suggest that methemoglobinemia is not a prerequisite for hemolysis.

Porphyria

At least three different types of experimental porphyria can be induced by drugs and other foreign compounds (110): 1. That produced by allyl ureide and allyl acetamide compounds, such as sedormid, allylisopropylacetamide, and secobarbital; 2. that caused by hexachlorobenzene; 3. that caused by dicarbethoxydihydrocollidine and related compounds. In addition to causing porphyria and increasing the synthesis of δ-aminolevulinic acid, the allyl compounds, such as allylisopropylacetamide (111, 112), secobarbital, allobarbital and aprobarbital (112) that produce porphyria, also catalyze the destruction of the various heme compounds in liver, especially cytochrome P-450. Recently, it was shown that a number of other allyl compounds, including allyl alcohol, acrylamide, and the anesthetic gas, fluo
a selective destruction of liver microsomal cytochrome P-450 (23). The finding that these effects are markedly increased after pretreatment of rats with phenobarbital suggest that they are caused by active metabolites of the foreign compounds. In support of this view, secobarbital decreases the cytochrome P-450 in liver microsomes only when NADPH is present in the incubation system (112). In contrast to the destructive effects of compounds such as CCl_4 (66) on cytochrome P-450, the effects of secobarbital are not blocked by EDTA (112). Thus, the effects probably are not mediated by lipid peroxidation. Because allyl compounds are converted to epoxides, it seems possible that these might be the active metabolites. However, this possibility has not been confirmed, and it is not known whether the active metabolites bring about their action by becoming covalently bound to cytochrome P-450 or to other macromolecules.

The destruction of heme compounds in liver may be an initial event in the development of porphyria (111). Because heme is known to exert a negative feed-back control on the conversion of succinyl-CoA to δ-aminolevulinic acid, the rate-limiting step in heme synthesis, any mechanism that decreases the level of the intracellular heme pool that exerts the feed-back control would be expected to increase the rate of porphyrin synthesis. However, the rate-controlling pool of heme probably is not cytochrome P-450, because the heme in cytochrome P-450 does not exchange with free heme (113). The finding that heme in the mitochondrial and the soluble fractions of liver is also destroyed by allylisopropylacetamide (111) raises the possibility that a heme pool in these fractions may serve to control porphyrin synthesis.

Hypersensitivity Reactions

The mechanisms by which drugs cause allergic responses remains largely unexplored. Since the classic work of Landsteiner and his colleagues during the 1920s (114), it has been shown by many investigators that small molecules can serve as antigens only after they become covalently bound to macromolecules, such as plasma albumin (115). Consequently, the finding that drugs can be converted to chemically reactive metabolites that combine covalently to macromolecules raised the possibility that the formation of chemically reactive metabolites might be an initial event in drug-induced hypersensitivity reactions. Indeed the failure to demonstrate covalent binding of drugs to macromolecules either in vivo or in vitro might suggest that the drug would not evoke hypersensitivity reactions in man. On the

other hand, it has become evident that covalent binding of the drug does not always lead to antibody formation or an immunologic response. Indeed, investigators rarely are able to find antibodies to a given drug either in patients manifesting hypersensitivity reactions or in animals receiving a drug that is known to be covalently bound to macromolecules.

PHARMACOKINETICS AND OTHER CONSIDERATIONS

Inhibitors and inducers of drug metabolizing enzymes have obviously played an important role in determining whether a compound evokes its pharmacologic and toxic responses by itself or through the formation of an active metabolite. It is not always realized, however, that the finding that inhibitors such as SKF 525-A increase the response or that inducers such as phenobarbital decrease the response does not preclude the possibility that a toxin exerts its effect through the formation of a chemically reactive metabolite (23). Moreover, if the rate of metabolic activation of a toxin is kinetically a first order reaction, increasing or decreasing the rate of formation theoretically should not change the total amount of toxin produced, assuming that the elimination of the compound from the body is primarily dependent on its metabolism through the toxic pathway. Most substances, however, are eliminated by competing or coupled metabolic reactions in the liver as well as by elimination via the kidneys, lungs, or biliary system. Thus, with drugs that are eliminated from the body largely unchanged, treatments that change the rate of conversion of the parent drug to the chemically reactive metabolite would be expected to alter the amount of metabolite formed even though the treatments do not affect the biological half-life of the drug. On the other hand, with drugs that are largely metabolized along a number of different pathways, the effect of the treatments on the total amount of chemically reactive metabolite formed would depend on whether the treatments changed the relative importance of the different pathways of metabolism. When the treatments increase or decrease the rates of metabolism along the different pathways to the same extent, the total amount of chemically reactive metabolite formed would not be changed. It is also important to realize that treatments can alter the fate of the chemically reactive metabolites and thereby affect the amounts of the metabolites that become covalently bound.

These principles may be illustrated by a number of examples of the covalent binding of drugs to liver macromolecules; 1. Although pretreatment of mice with phenobarbital markedly increases the rate of bromobenzene metabolism both in vivo and in vitro, it does not appreciably affect the amount of a subtoxic dose that becomes covalently bound to liver proteins (76), because almost all of the toxicant is converted to 3,4-bromobenzene epoxide in both untreated and pretreated animals. 2. In contrast, pretreatment of rats with 3-methylcholanthrene prevents the hepatic damage produced by bromobenzene (16, 116) and 2-acetylaminofluor
parently by stimulating detoxifying metabolic pathways more than toxifying ones. 3. The major pathways by which acetaminophen is eliminated is not through the formation of its chemically reactive metabolite but through the formation of its conjugates with glucuronic acid and sulfate (40). Consequently, the covalent binding

and hepatotoxicity of acetaminophen in mice is increased by phenobarbital and decreased by cobaltous chloride, even though these treatments have no significant effect on the half-life of the drug in mice (26). 4. Dibenamine decreases the toxicity of CCl_4 by preventing its conversion to the free radical and chloroform, yet dibenamine has no effect on the half-life of CCl_4 (49), because most of the toxicant is eliminated via the lungs. 5. The proportion of the dose of furosemide that becomes covalently bound to liver protein increases as the dose is increased above a threshold, apparently because the clearance of the drug from the body is decreased as the dose is increased (38). 6. When doses of bromobenzene or acetaminophen that deplete the liver of GSH are used, pretreatment of animals with phenobarbital increases the covalent binding (13, 14, 23, 34) because the rate of formation of the GSH conjugate is limited by the rate of formation of GSH rather than by that of the epoxide. 7. Depletion of sulfate decreases the toxicity of 2-acetylaminofluorene because the formation of the chemically reactive N-hydroxy sulfate conjugate of the carcinogen is decreased (3, 4) relative to the other pathways of elimination of the toxicant. On the other hand, depletion of hepatic GSH by diethyl maleate increases the toxicity of the halobenzenes and acetaminophen (13, 14, 19, 23, 34) by decreasing the formation of the nontoxic GSH conjugates of these substances. In addition, diethyl maleate increases the covalent binding and toxicity of CCl_4 and furosemide even though these compounds themselves do not decrease GSH levels in liver. Thus, nontoxic substances can affect the severity of the injury caused by toxicants even through the nontoxic substance is neither an "inducer" nor an "inhibitor" in the usual sense of these words.

The kinetic aspects of chemically reactive metabolites are even more complex when the metabolites are formed in extrahepatic tissues as well as in the liver. They depend on whether the chemically reactive metabolite can escape the liver and then be carried to the extrahepatic tissue by the blood. If the metabolite is so chemically reactive that it cannot escape the hepatocyte in which it is formed, then pretreatment of animals with substances that induce the liver enzymes but not the extrahepatic tissues would be expected to decrease toxicity in the extrahepatic tissues. Moreover, substances that specifically inhibit the extrahepatic enzymes but not the liver enzymes would also be expected to decrease toxicity in the extrahepatic tissues. However, inducers and inhibitors of drug metabolizing enzymes are seldom specific, and their effects on extrahepatic enzymes are incompletely understood. For these reasons, it often is not possible to obtain unequivocal information that would differentiate among the various mechanisms for the toxicity of drugs in extrahepatic tissues.

CONCLUSIONS

In most studies of the mechanisms of chemical toxicity, emphasis has been placed on the use of toxic substances as metabolic probes of cellular function (118). When the toxin brings about its effect by interacting with a specific biochemical system, such studies are useful approaches to an understanding of how the biological system responds to such metabolic rearrangements. But when the toxins interact with a number of biochemical systems simultaneously, as chemically reactive metabolites

frequently do, it is difficult to determine whether changes in cell function result from a sequence of changes originating from a single initial biochemical alteration or from the concerted action of a number of different initial biochemical alterations. In any event, such studies frequently fail to provide any practical information about the nature of the active form of the toxin or any clues to methods for preventing the toxicity.

From studies with liver microsomes in vitro, it is now clear that many drugs can be converted to chemically reactive metabolites because they become covalently bound to microsomal protein in incubation systems containing liver microsomes, NADPH, and the drug. But it is not clear to what extent the covalent binding can be used to predict the incidence of various kinds of toxicity in animals or man. Nevertheless, such in vitro studies might be useful in choosing which of a series of analogs having similar pharmacologic activity should receive the highest priority in the development of drugs, particularly when the members of the series also lead to widely different amounts of covalently bound metabolites in vivo.

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