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Toxicological Implications of Drug Metabolism

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Normally the duration and intensity of drug action are related to the rate at which the drug is eliminated from the body. In most cases hepatic and renal clearances, as well as tissue binding, are limiting factors in the determination of total body clearance. Most lipophilic drugs and foreign compounds are biotransformed by a variety of enzymatic pathways to more polar and readily excretable metabolites. Although many extrahepatic tissues, particularly the lung, have been shown to metabolize drugs, steroids, and xenobiotics, the liver is the major organ involved in the biotransformation of foreign compounds. This brief review discusses the biochemical processes in liver most actively involved in generating more hydrophilic metabolites from less polar foreign compounds and their toxicological and pharmacological implications.

The enzymes involved in the biotransformation of foreign compounds are embedded in membranes of the endoplasmic reticulum of liver cells. The endoplasmic reticulum, a network of interconnected channels throughout the cytoplasm, exists in two forms, respectively rough and smooth membranes, which differ in both form and function. The rough membranes are studded with ribosomes, which translate genetic information into sequences of amino acids, while the smooth portion of the endoplasmic reticulum, relatively devoid of ribosomes, is rich in drug metabolizing enzymes that proliferate during chronic exposure to drugs and a variety of foreign substances. This process of proliferation, referred to as liver microsomal enzyme induction, is frequently involved in the altered pharmacological and toxicological responses to drugs. Unfortunately, it is not possible to study the biochemical properties of the endoplasmic reticulum as one functional unit. To separate the membranes, the cell must be fractured by homogenization and subjected to high speed differential centrifugation (27). The remaining pellet, referred to as the microsomal fraction of the hepatocyte, contains the mixed function oxidases that metabolize drugs and foreign compounds. Table 1 lists the types of reactions catalyzed by these mixed function oxidases. Many types of oxidations have been described, including alkyl chain oxidation to an alcohol, aromatic ring hydroxylation to phenols, N- and O-dealkylation, deamination of amphetamine, N-hydroxylation to form hydroxamic acid, epoxidation and hydration to diols as well as a variety of reductions, hydrolyses, and conjugations. The fact that these enzymes are localized in the smooth portion of the endoplasmic reticulum and require both molecular oxygen and the reduced form of the pyridine nucleotide (NADPH) has led biochemists to classify the system as a mixed function oxidase (5). The general scheme for the transfer of electrons from NADPH to the drug substrate is shown in figure 1. A flavoprotein, NADPH-cytochrome P-450 reductase, is first reduced, then electrons are transferred to cytochrome P-450 (3). Omura and Sato (21,22) first called the cytochrome P-450 because carbon monoxide forms a complex with the reduced form of the cytochrome, which then absorbs light maximally at 450 nm. The reduced (Fe^{++}) form of P-450 reacts with molecular oxygen in such a way

TABLE 1
Liver microsomal drug metabolism

Drug	Type	Product
	Oxidation	
Pentobarbital	Side chain	Alcohol
Phenobarbital	Ring hydroxylation	Phenol
Bromobenzene	Epoxidation	Diols, phenol
Methadone	N-dealkylation	Secondary amine
Codeine	O-dealkylation	Phenol
Amphetamine	Deamination	Ketone
Acetylaminofluorene	N-hydroxylation	Hydroxamic acid
Chlorpromazine	S-oxidation	Sulfoxides
	Reduction	
Prontosil	Azo reduction	Sulfanilamide
Chloramphenicol	Nitro reduction	Aromatic amine
	Hydrolysis	
Procaine	Esterase	Acid
Cinnamamide	Amidase	Acid
	Conjugation	
Salicylic acid	Esterification	Glucuronide
Sulfanilamide	Acylation	Amide
Acetaminophen	Sulfation	Mercapturic acid

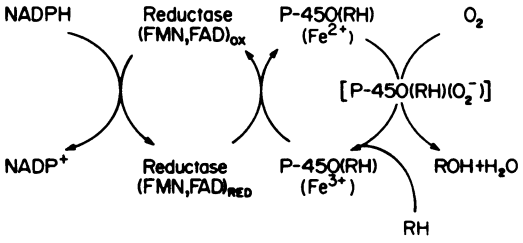

FIG. 1. NADPH-cytochrome P-450 electron transport system.

that one of the oxygen atoms is reduced to water and the other is introduced into the organic substrate. The presence of phosphatidylcholine appears to be necessary for the rapid flow of electrons from NADPH to cytochrome P-450 (29). The formation of a complex that contains cytochrome P-450, substrate, and superoxide anion is shown as a possible step in the formation of the hydroxylated substrate and water (3).

Cytochrome P-450, which acts as terminal oxidase, increases greatly in concentration in the liver of animals exposed to certain drugs, chemicals, and environmental pollutants (2). The stimulation of synthesis of P-450 results in the proliferation of the smooth portion of the endoplasmic reticulum of liver (7,23). This proliferation has been regarded as a defensive or protective adaptation that leads to enhanced clearance of the foreign compound. An example of the induced system after the administration of phenobarbital to rats is shown in figure 2. Rats were treated with phenobarbital for 3 days. On the 4th day cytochrome P-450 and ethylmorphine N-demethylase were measured in vitro, while the disappearance of antipyrine from plasma was determined in vivo. As can be seen, P-450 and ethylmorphine N-demethylase activity were greatly increased in vitro and the rate of elimination of antipyrine from plasma was enhanced, reflecting increased metabolism in vivo. The nature of the cytochrome induced by drugs and environmental agents may vary according to the inducing agent. For example, exposure of rats to carcinogenic substances such as the polycyclic hydrocarbons, benzo(a)pyrene, benzanthracene, and methylcholanthrene results in the induction of a different cytochrome (P-448) with different substrate specificities. Figure 3 shows the effect on the liver microsomal cytochrome of treating rats with methylcholanthrene for 3 days. It is clear that there is a slight but significant shift in the CO-binding spectrum of the P-450 to a shorter wave length (13). This spectrally different cytochrome has catalytic proper-

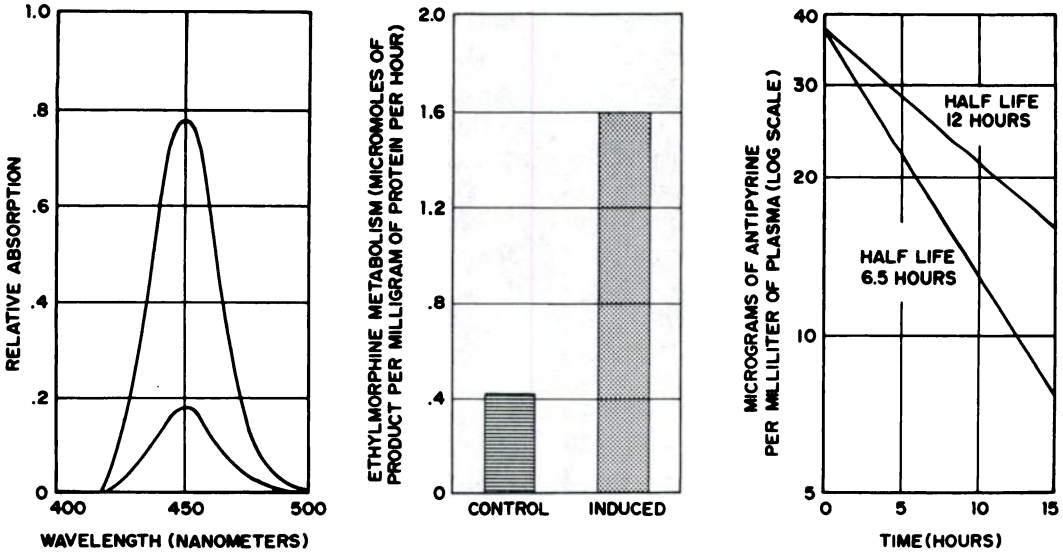

FIG. 2. Effect of pretreating rats with phenobarbital on liver microsomal cytochrome P-450, ethylmorphine N-demethylase activity, and on the in vivo plasma half-life of antipyrine.

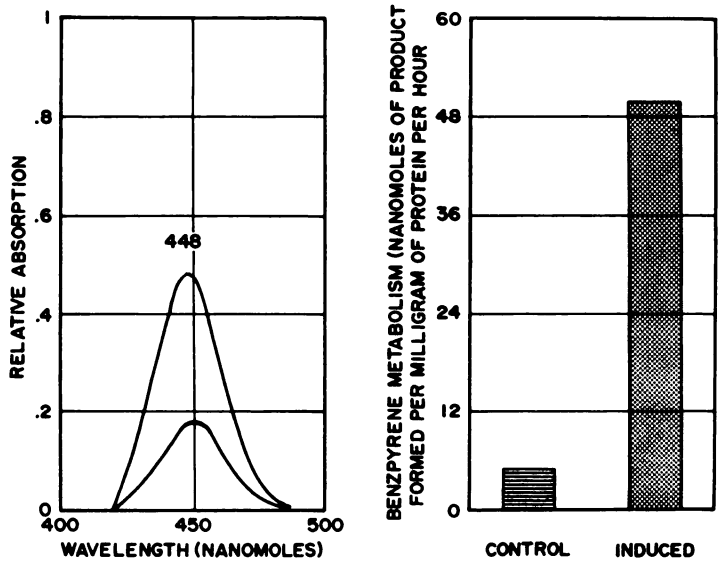

FIG. 3. Effect of pretreating rats with 3-methylcholanthrene on liver microsomal cytochrome P-450 and benzo(a)pyrene hydroxylase activity.

ties that favor the metabolism of benzo(a)pyrene (fig. 3) and other polycyclic aromatic hydrocarbons (14).

The observation that polycyclic aromatic hydrocarbons and certain other xenobiotics can induce the formation of a cytochrome with a different CO-binding spectrum suggested that P-450 may actually represent a mixture of cytochromes with different substrate specificities. This hypothesis was further supported by studies of the metabolism of the steroid hormone testosterone (32). Among other metabolites, testosterone is metabolized by the cytochrome P-450 system of liver microsomes to the 7α, 16α, and 6β hydroxylated metabolites.

Moreover, the formation of these metabolites may be differentially influenced by inhibitors and inducers of the microsomal system. Table 2 shows the effect of an insecticide, chlorthion, on the ability of isolated liver microsomes to hydroxylate testosterone in three different positions. Chorthion at 10^{-5} M inhibited the formation of 16α-hydroxytestosterone by 40% without affecting the formation of the 6β- or 7α-hydroxylated metabolites. At 10^{-4} M, this insecticide completely inhibited the formation of 16α-hydroxylated testosterone with only minimal effects on the other two metabolites. These results suggested that a mixture of cytochromes may exist in liver microsomes and triggered a 10-year effort to solubilize, isolate, and determine the substrate specificities for the different cytochrome P-450s.

In 1968, Lu and Coon and coworkers (15,29) first solubilized the liver microsomal hydroxylating system with the ionic detergent deoxycholate and chromatographically resolved the system on a DEAE-cellulose column into three components, which were identified as cytochrome P-450, NADPH-cytochrome *c* reductase, and a lipid fraction. Table 3 shows the need for all three components for the N-demethylation of benzphetamine, the 16α-hydroxylation of testosterone, and the metabolism of benzo(a)pyrene (16). Essentially no metabolite formation occurred in the absence of any one of these components. All three components have since been shown to be required for the metabolism of a variety of substrates, including drugs, chemical carcinogens, steroids, and fatty acids (17). The active lipid component has been identified as phosphatidylcholine and shown to be essential for electron transfer from NADPH to cytochrome P-450 (29). Since phosphatidylcholine is obviously not an electron carrier, the mode of action of this lipid remains unknown.

The successful resolution and reconstitution of the liver microsomal hydroxylating system has provided a means for separation of a number of cytochromes and for determination of their catalytic specificities. For example, different forms of P-450

TABLE 2

In vitro effect of chlorthion on the metabolism of testosterone by rat liver microsomes†*

Inhibitor	Conc. (M)	Hydroxylated Testosterone Formed			Total Polar Metabolite Formed (nmol)
		6 β-OH	7α-OH (nmol)	16α-OH	
Control		91	27	70	420
Chlorthion	10^{-4}	41	22	3	98
Chlorthion	10^{-5}	91	31	41	231

* *p*-Nitro-*m*-chlorophenyldimethylthionophosphate.

† Liver microsomes from adult male rats were prepared and incubated with testosterone-4-^{14}C in the presence of an NADPH-generating system. Chlorthion was added immediately before the substrate. (See Welch et al. (32) for details of assay method.)

TABLE 3

*Requirements for drug and steroid hydroxylation**

Substrate	Complete	Lipid	Reductase	Cytochrome P-450
Benzphetamine N-demethylation (nmol/min)	8.4	0.48	0	0.12
Testosterone 16 α-hydroxylation (nmol/17.5 min)	3.02	0.25	0	0
Benzo(a)pyrene hydroxylation (nmol/5 min)	2.28	0.42	0.02	0.04

* The complete system contained 1 nmol of cytochrome P-450 (for benzphetamine and testosterone), or 0.5 nmol of P-448 (for benzo(a)pyrene), 0.1 mg of lipid, 195 units of NADPH-cytochrome *c* reductase and other necessary cofactors. For further details see Lu and Levin (16).

are induced in the liver of rats pretreated with carcinogens, polychlorinated biphenyls, and barbiturates. The electrophoretic mobility, spectral characteristics, and catalytic properties of a mixture of hemeproteins isolated from microsomes of rats pretreated with either phenobarbital or 3-methylcholanthrene were determined by Ryan et al. (25). As shown in table 4, these authors could distinguish three distinct hemeproteins on sodium dodecyl sulfate-polyacrylamide gels with molecular weights of 44,000, 47,000, and 53,000. Phenobarbital preferentially induced the hemeprotein with a MW of 47,000 and 3-methylcholanthrene favored the induction of the 53,000 MW hemeprotein. A hemeprotein with a MW of 44,000 was present in all fractions. More recently Ryan et al. (26) have been able to purify and further characterize these cytochromes. The carbon monoxide binding characteristics of these hemeproteins produced different spectral peaks; the peak CO difference spectrum for P-450a occurred at 452 nm, while those for P-450b and P-450c occurred at 450 nm and 447 nm, respectively. Moreover, these different cytochromes have different substrate specificities, as shown in table 5. The catalytic activity was determined in the presence of saturating amounts of NADPH-cytochrome P-450 reductase and phosphatidylcholine. As shown from the relative turnover numbers, the purified cytochrome P-450b from phenobarbital-treated rats preferentially catalyzed benzphetamine N-demethylation and testosterone 16α-hydroxylation. The hemeprotein, P-450c, from 3-methylcholanthrene-treated rats metabolized zoxazolamine, benzo(a)pyrene, and 7-ethoxycoumarin more efficiently than the phenobarbital-induced cytochrome P-450. In addition, immunochemical experiments (30) in which antibodies against purified rat liver cytochromes P-450 and P-448 were used have provided evidence for six forms of rat liver cytochrome P-450. Although the catalytic activities of these immunologically different hemeproteins remain to be determined, their roles in drug metabolism will most likely be of significance for toxicology.

TABLE 4
*Physical properties of various forms of cytochrome P-450**

Cytochrome	Inducer	Co-difference, Spectral Peak	MW (SDS Gels)
P-450a		452 nmol	44,000
P-450b	Pb	450 nmol	47,000
P-450c	3-MC	447 nmol	53,000

* Data taken from Ryan et al. (26).

TABLE 5
Catalytic activity of various purified forms of rat liver cytochrome P-450

Substrate*	Phenobarbital		3-Methylcholanthrene	
	P-450a	P-450b	P-450a	P-450c
Benzphetamine	2.2	216.6	2.6	5.0
Benzo(a)pyrene	0.04	0.2	0.3	24.5
7-Ethoxycoumarin	0.2	13.9	1.1	67.5
Zoxazolamine	0.48	2.20	2.0	29.7
Testosterone				
7α-OH	4.1	0.06	5.4	0.08
16α-OH	0.08	1.3	0.04	0.02
6β-OH	0.11	0.04	0.08	0.36

* Activity expressed as nanomoles of product formed/min/mole of cytochrome P-450. Data taken from Ryan et al. (26).

More recently considerable attention has been given to the fact that many drugs and xenobiotics are metabolized in the body by the P-450 mixed-function oxidase system into compounds that may evoke therapeutic or toxic responses or both. Certain drugs owe their therapeutic activity to a metabolite produced by the mixed-function oxidase system. For example, codeine is in part directly conjugated to form a glucuronide or sulfate and in part metabolized by N- and O-demethylation and then conjugated. One of the metabolites of codeine, morphine, is a potent analgesic and is most likely responsible for the analgesic activity of codeine. Only recently have methods been developed to measure plasma levels of morphine in man after the usual analgesic dose of codeine (4). Figure 4 shows the disposition of codeine and morphine in man after the oral administration of 60 mg of codeine contained in a commonly used an-

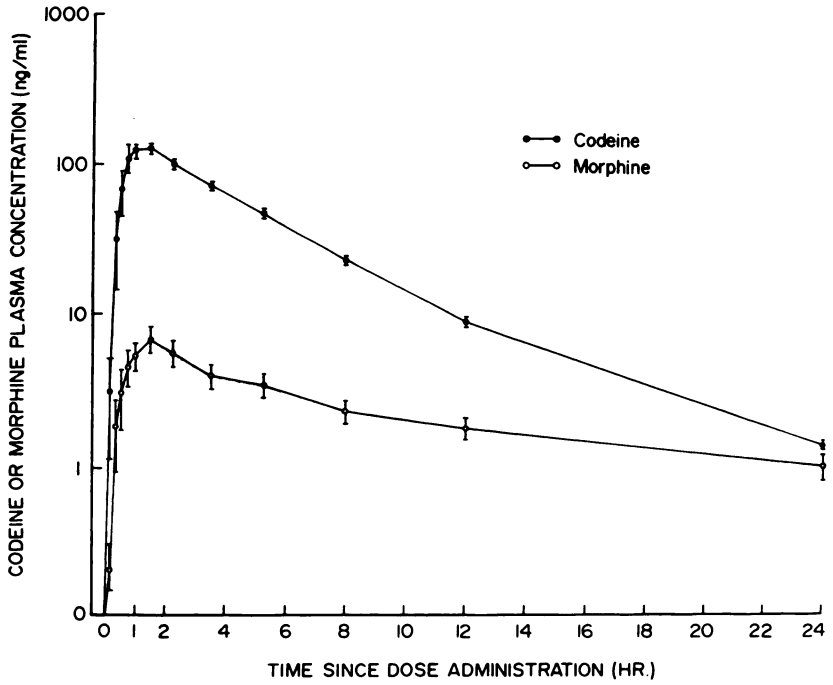

FIG. 4. Codeine and morphine plasma concentrations in 12 subjects following oral administration of aspirin-codeine phosphate combination (mean ± S.E.M.). See Findlay et al. (4) for further details.

algesic mixture. As can be seen, plasma levels of morphine could be measured for 24 hours, which represented about 10% of the area under the codeine plasma curve. Interestingly, it has been reported that codeine, on a weight basis, has about 10% of the analgesic potency of morphine in man (31). Since codeine undergoes O-demethylation to morphine by action of the mixed-function oxidase system, codeine may be regarded as a pro-drug that provides sufficient morphine for analgesia without producing high plasma levels of morphine associated with euphoria and addiction.

In some instances, drugs and other foreign compounds may be metabolized in the body by the NADPH-cytochrome P-450 system to chemically reactive metabolites that either uncouple integrated biochemical processes in cells or combine covalently with various tissue macromolecules such as DNA, RNA, and protein. During the past several years it has become increasingly evident that chemically reactive metabolites mediate many different kinds of serious toxicity, including carcinogenesis, mutagenesis, and cellular necrosis. Ames (1) has developed a simple, inexpensive, and very sensitive test for the detection of chemical compounds that are or become mutagenic. The detection of mutations involves a simple back mutation test: the reversion from histidine requirement in *Salmonella typhimurium* auxotrophs to growth on media deficient in histidine. As shown in table 6, mutagens such as aflotoxin B_1, benzopyrene-7,8-dihydrodiol, and acetylaminofluorene become mutagenic only when the test system contains the hepatic NADPH cytochrome P-450 system (18,33). Aflotoxin B_1, and benzo(a)pyrene-7,8-dihydrodiol have been shown to be oxidized by the microsomal fraction of liver to epoxides that are the ultimate carcinogens.

In fact, reactive epoxides of many drugs form the molecular basis for their toxicity. One of the most extensively studied chemicals that undergoes epoxidation to a reactive electrophile is bromobenzene. In vivo and in vitro studies on the metabolism and

covalent binding of bromobenzene (24) by rat liver have permitted a direct evaluation of the relative importance of certain metabolic pathways (fig. 5). Bromobenzene undergoes activation by the cytochrome P-450 system to the electrophile, 3,4-bromobenzene epoxide. Depending on the concentration of this epoxide, it may undergo rearrangement nonenzymically to bromophenol, hydration by epoxide hydrase to yield the water soluble dihydrodiol, or conjugation with glutathione. The glutathione conjugate is further metabolized to yield finally the urinary metabolite bromophenylmercapturic acid. Glutathione-S-epoxide transferase is a soluble enzyme in the cytosol and is the preferred detoxication pathway for bromobenzene epoxide (8). How-

TABLE 6
Hepatic activation of carcinogens

Carcinogen	Carcinogen Added, μg	Liver Supernatants, 9000 × g	Histidine Revertants per Plate*
2-Acetylaminofluorene	0	+	46
	50	−	21
	50	+	13,600
Benzo(a)pyrene	0	+	44
	5	−	34
	5	+	505
Aflatoxin	0	+	26
	1	−	26
	1	+	266

* The histidine-requiring Salmonella mutant TAI538 was incubated with carcinogen for 2 days in the presence and absence of a rat liver 9000 × *g* supernatant fraction and an NADPH-generating system. The colonies on the plates (HIS^+ revertants) were counted after a 2-day incubation at 37°C in the absence of histidine. Data taken from McCann et al. (18) and Wood et al. (33).

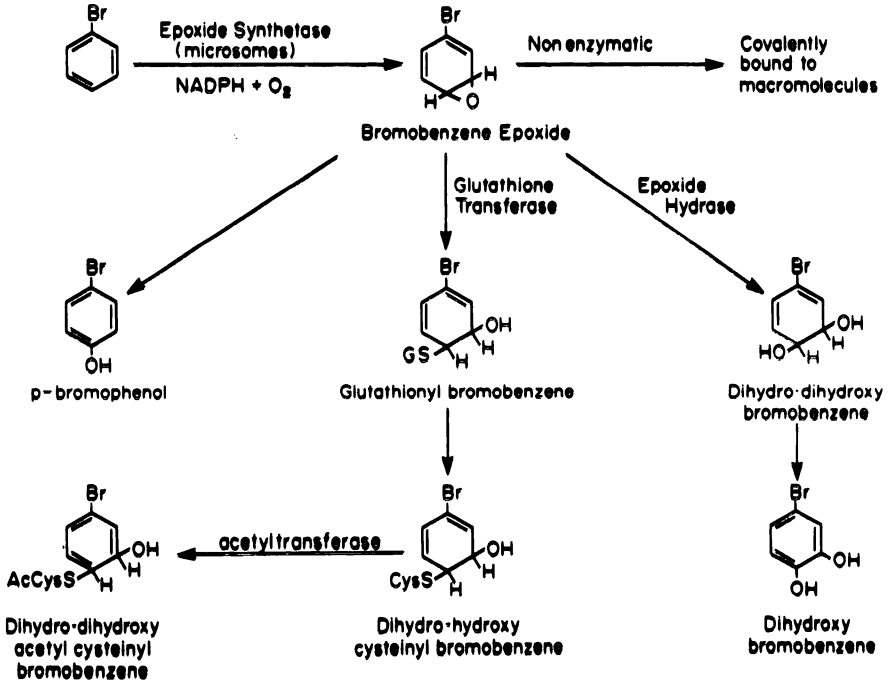

FIG. 5. Pathways of bromobenzene metabolism (Taken by permission from Jollow et al. (8)).

ever, if glutathione becomes depleted, the glutathione transferase-catalyzed pathway becomes rate-limiting, allowing the concentration of bromobenzene epoxide to rise, which results in significant covalent binding of this electrophile to protein and other electron-rich macromolecules. Such a process generally leads to cell death and can explain the centrolobular hepatic necrosis observed in the liver of rats treated with bromobenzene. It is also interesting to note that histochemical evidence indicates that cytochrome P-450 enzymes in the endoplasmic reticulum of central hepatocytes are especially active in metabolizing drugs and foreign compounds (12).

The commonly used analgesic, acetaminophen, is generally considered safe at normal therapeutic doses but it is quite hepatotoxic at high doses ingested accidentally or with suicidal intent. In animals treated with high doses of acetaminophen there is an obvious lack of correlation between acetaminophen tissue levels and acetaminophen-induced hepatic necrosis, which suggests that a toxic metabolite(s) of acetaminophen is responsible for the hepatocellular damage (19). The metabolic pathway shown in figure 6 has been suggested to explain the hepatotoxicity of acetaminophen (20). Although the major portion of the acetaminophen dose is conjugated with sulfate and glucuronic acid to form water-soluble, readily excreted metabolites, a significant portion of the dose is N-hydroxylated, particularly at high doses that saturate the conjugating pathways. The N-hydroxyacetaminophen is unstable and readily dehydrates to form a chemically reactive imidoquinone. When an adequate concentration of glutathione is present, this reactive metabolite preferentially reacts with glutathione to form a nontoxic conjugate that is ultimately excreted in urine as a mercapturic acid. If, however, the liver becomes depleted of glutathione because of dietary deficiency or because a large dose of acetaminophen was administered, the imidoquinone combines with essential macromolecules to cause centrolobular hepatic necrosis.

The relationships between the dose of acetaminophen, glutathione levels in liver, covalent binding, and hepatic necrosis, as described by Gillette and Mitchell (6), are shown in table 7. Mice treated with doses

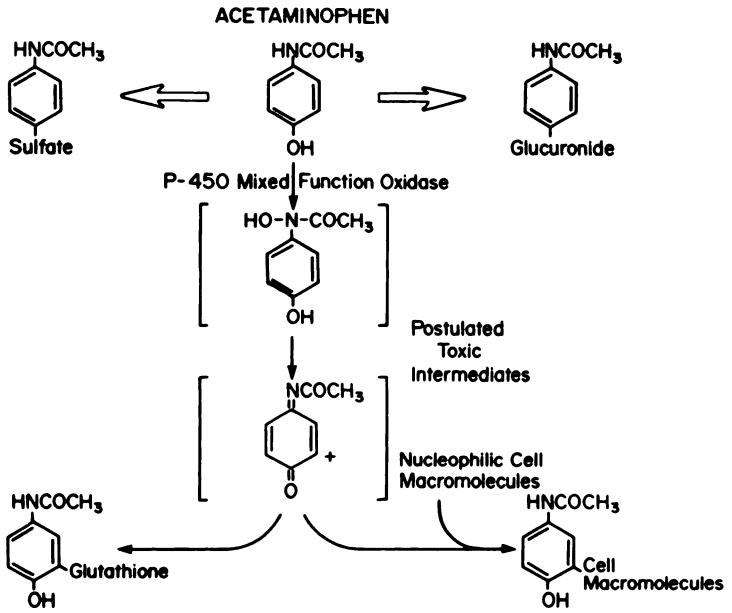

FIG. 6. Pathways of acetaminophen metabolism (Taken from data of Mitchell et al. (20)).

TABLE 7

*Relationship between glutathione levels in liver, covalent binding of acetaminophen metabolites, and liver necrosis in mice**

Dose of Acetaminophen (mg/kg)	Hepatic Glutathione Per Cent of Initial Level	Covalent Binding (nmol/mg protein)	Toxicity
100	76	0.04	None
200	41	0.08	None
375	19	0.71	Minimal
750	17	1.89	Extensive

* Data taken from Mitchell et al. (19).

of radiolabeled acetaminophen below 375 mg/kg showed no significant hepatotoxicity and insignificant covalent binding of radiolabel even when glutathione in the liver was depleted by as much as 60%. However, when the dose of acetaminophen was increased to 375 mg/kg and above, the glutathione levels declined to 20% of normal, covalent binding increased significantly, and hepatotoxicity became apparent.

Compounds such as diethyl maleate that deplete glutathione markedly enhance the hepatotoxicity induced by electrophilic metabolites formed from a variety of drugs. In fact, depletion of glutathione by diethyl maleate may be a useful toxicological tool for the early detection of toxic reactive metabolites of drugs. For example, as shown in table 8, acetaminophen at 375 mg/kg caused minimal hepatotoxicity in mice associated with some degree of covalent binding to liver protein; but mice pretreated with diethyl maleate 30 min prior to the same dose of acetaminophen showed marked covalent binding associated with severe liver necrosis. Moreover, the concomitant administration of cysteine protected mice from the covalent binding and the hepatotoxicity induced by acetaminophen. This protective action of cysteine is best explained by its role as a precursor for the synthesis of glutathione.

An understanding of the metabolic pathway involved in the generation of electrophilic metabolites is useful in designing compounds with the desired pharmacological effects but devoid of potential toxicity. Recent reports have described an interesting interaction between phenacetin and acetaminophen (9,28). Table 9 shows that liver glutathione was depleted and the mortality of mice was increased following the oral administration of either 300 mg/kg or 600 mg/kg of acetaminophen. However, similar doses of phenacetin produced no mortality and had little effect on the glutathione concentration in liver. Moreover, the concomitant administration of phenacetin along with normally toxic doses of acetaminophen markedly reduced the mortality produced by acetaminophen. The protective effect of phenacetin against acetaminophen-induced hepatotoxicity is probably best explained by the ability of phenacetin to compete effectively for N-hydroxylation with acetaminophen (11). When phenacetin is coadministered with acetaminophen, there is competition for the N-hydroxylating pathway, which reduces the amount of N-hydroxyacetaminophen formed. Although this may lead to the for-

TABLE 8

*Effect of diethyl maleate or cysteine on in vivo covalent binding of acetaminophen (375 mg/kg) to mouse liver protein**

Treatment†	Severity of Liver Necrosis (after PHAA)	Protein Bound Acetaminophen (nmol/mg protein/liver)
None	Minimal	0.88 ± 0.09
Diethyl maleate	Extensive	1.57 ± 0.11
Cysteine	None	0.38 ± 0.08

* Data taken from Gillette et al. (6).

† Diethyl maleate 0.3 ml/kg, i.p., 30 min before acetaminophen; cysteine 150 mg/kg, i.p., 5 min before and 20 min after acetaminophen.

TABLE 9
Inhibition of acetaminophen-induced hepatotoxicity by phenacetin in the mouse†*

Compound	Dose (mg/kg)	Glutathione Depletion	Mortality (%)
Acetaminophen	300	90	30
Acetaminophen	600	92	80
Phenacetin	300	30	0
Phenacetin	800	30	0
Phenacetin + acetaminophen	300 + 300	20	0
Phenacetin + acetaminophen	300 + 600	40	0

* Male mice (C57BL/6J) received the drugs i.p. Glutathione levels were measured 1 hr after the dose and mortality was determined after 3 days.

† Data taken from Kapetanovic and Mieyal (9,10).

mation of more N-hydroxyphenacetin, this metabolite is relatively nontoxic (10).

The above studies on the formation of reactive metabolites stress the need for a better understanding of the major pathways involved in drug metabolism for the proper interpretation of toxicological data.

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