ABSTRACT Treatment of rats with phenobarbital, which stimulates the activity of the drug-metabolizing enzymes in the liver, potentiates hepatic necrosis elicited by bromobenzene and a number of other chemically inert halogenated aromatic hydrocarbons. Radioautographic studies indicate that $^{14}C$ bromobenzene is covalently bound at the sites of necrosis. From these results, it is inferred that the hepatotoxic effects of the halogenated aromatic hydrocarbons are mediated by chemically active metabolites formed in hepatocytes. In accord with this view, a number of aromatic halogenated hydrocarbons are converted by microsomes in vitro to active intermediates which form covalent complexes with glutathione (GSH).

Although drugs are generally converted in the body to derivatives that are less toxic than the parent compounds, many therapeutic agents occasionally produce tissue damage, such as hepatic and renal necrosis, blood dyscrasias, and other lesions. Some years ago Brodie suggested (1) that chemically inert therapeutic drugs may cause tissue lesions by the formation of a covalent linkage between a chemically active metabolite and various macromolecules. A similar mechanism has been invoked to account for the carcinogenic activity of chemically inert substances, such as dialkyl nitrosamines, azo dyes, N-acetylaminofluorene, and polycyclic hydrocarbons (2). To test the view that other tissue lesions caused by chemically stable organic compounds might be mediated through active metabolites, we have investigated the toxic mechanisms of bromobenzene and other aromatic hydrocarbons that are known to cause liver necrosis in various animal species (3, 4).

METHODS

Histologic studies in vivo

Male Sprague-Dawley rats (NIH, about 180 g) were injected for three successive days with saline or with phenobarbital (80 mg/kg, i.p.). On the fourth day, three groups of four animals each were injected with various doses of organic compounds (i.p. in 0.5–2 ml of sesame oil). 24 hr later, the livers were removed and slices 1–2 mm thick were fixed in buffered formalin, dehydrated in graded strengths of ethanol, cleared in xylol, and embedded in paraffin. Sections (6 μm thick) were stained with periodic acid–Schiff reagent (PAS) and hematoxylin (5). The PAS reagent stains glycogen in the cytoplasm of the parenchymal cells and hematoxylin counterstains the nuclei. Pathological changes in the hepatocytes in the centrolobular region were arbitrarily classified as follows: no observable change; no necrosis but a loss of glycogen; a minimal necrosis (a few degenerating parenchymal cells); extensive necrosis (central veins were surrounded by several rows of dead or degenerating cells); and massive necrosis of extensive liver areas.

Autoradiographic studies

An untreated mouse (male, NIH strain) was given $^{14}C$ bromobenzene (0.013 ml/30 g, 250 μCi, in 0.4 ml of sesame oil, i.p.) and killed 40 hr later. The liver was removed and paraffin sections were prepared as described above. The sections were stained with PAS and coated with Kodak NTB-2 emulsion. The radioautogram was put into a light-free box for 2 weeks at −20°C, then developed and finally counterstained with Harris’s hematoxylin (6).

In vitro formation of glutathione (GSH) conjugates

Highly electrophilic metabolites (alkylating agents) formed from the aromatic hydrocarbons were trapped by allowing them to form a covalent bond with GSH, a potent nucleophile. Various compounds were incubated with GSH plus dialyzed hepatic soluble fraction containing GSH transferase activity, liver microsomes from normal or phenobarbital-treated rats, and a NADPH-generating system for 1 hr at 37°C (7). The final concentrations in the incubation mixture (final volume 2.5 ml) were as follows: 0.5–30 mM hydrocarbon, 3 mM [35S]GSH (1 μCi/tube), 0.124 mM NADH, 2 mM nicotinamide, 0.2 mM NADP, 2 mM glucose-6-phosphate, 1 unit/ml glucose-6-phosphate dehydrogenase, 4 to 10 mg protein/ml liver microsomes, and 0.2 ml/ml of the soluble fraction. After the incubation, the hydrocarbon-GSH conjugates were separated by adsorption onto charcoal followed by paper chromatography (7).

RESULTS

Histologic studies

A number of halogenated compounds, many of which are listed in Table 1, produced necrosis of centrolobular parenchymal cells in liver. In a typical experiment, the histology of a normal liver (Fig. 1A) was compared with that of a liver from a rat injected with bromobenzene (0.2 ml) 24 hr previously (Fig. 1B). It is evident that bromobenzene caused extensive necrosis of parenchymal cells in centrolobular areas. A lower dose of bromobenzene (0.03 ml) caused, in these areas, a de-
crease in glycogen and some round cell infiltration but only minimal necrosis of hepatocytes (Fig. 1C).

Prior treatment of animals with phenobarbital markedly increased the centrolobular hepatotoxicity of a number of aromatic hydrocarbons. For example, a low dose of bromobenzene (0.03 ml), which caused little toxicity in untreated animals (Fig. 1C), produced extensive centrolobular necrosis in phenobarbital-treated rats (Fig. 1D). Phenobarbital administration also markedly increased the toxicity of chlorobenzene, iodobenzene, chlorinated naphthalenes, and 1,2- and 1,3-dichlorobenzences (Table 1). However, benzene and 1,4-dichlorobenzene did not produce liver lesions either in normal or in phenobarbital-treated rats.

**Studies with [14C]bromobenzene**

48 hr after the administration of 0.2 ml of [14C]bromobenzene (50 μCi) to a rat, considerable amounts of radioactivity were firmly bound in the liver. When a 3-mm-thick slice of liver (0.7 g) previously fixed in buffered formalin was homogenized in 10 volumes of buffered formalin, the homogenate centrifuged, and the pellet extracted with 10 ml of 50% ethanol and then with absolute ethanol, about 80% of the radioactivity (2600 dpm/g) remained with the pellet. In contrast when [14C]bromobenzene was added directly to the homogenate of a formalin-fixed slice of normal liver, most of the radioactivity was removed by two extractions with ethanol. These preliminary studies suggest that most of the radioactivity in the slice was covalently bound.

The autoradiogram in Fig. 2 shows extensive centrolobular necrosis in the liver of a 30-g mouse 40 hr after injection of 0.013 ml (250 μCi) of [14C]bromobenzene. The small black grains correspond to [14C]bromobenzene covalently bound to parenchymal cells despite extraction of the tissue in graded strengths of alcohol and xylol during preparation of the slide.

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**Table 1. Effect of phenobarbital administration on centrolobular necrosis**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose per rat</th>
<th>Control rats</th>
<th>Phenobarbital-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromobenzene</td>
<td>0.03 ml</td>
<td>Glycogen loss to minimal necrosis</td>
<td>Extensive necrosis</td>
</tr>
<tr>
<td>Iodobenzene</td>
<td>0.02 ml</td>
<td>Normal to minimal necrosis</td>
<td>Extensive to massive necrosis</td>
</tr>
<tr>
<td>Chlorobenzene</td>
<td>0.04 ml</td>
<td>Glycogen loss to minimal necrosis</td>
<td>Extensive to massive necrosis</td>
</tr>
<tr>
<td>Fluorobenzene</td>
<td>0.20 ml</td>
<td>No specific lesions</td>
<td>Extensive to massive necrosis</td>
</tr>
<tr>
<td>1,2-Dichlorobenzene</td>
<td>0.03 ml</td>
<td>Glycogen loss to minimal necrosis</td>
<td>Glycogen loss to massive necrosis</td>
</tr>
<tr>
<td>1,3-Dichlorobenzene</td>
<td>0.03 ml</td>
<td>Normal to minimal necrosis (rarely)</td>
<td>Glycogen loss to extensive necrosis</td>
</tr>
<tr>
<td>α-Chloronaphthalene</td>
<td>0.04 ml</td>
<td>Little or no effect</td>
<td>Minimal to extensive necrosis</td>
</tr>
<tr>
<td>β-Chloronaphthalene</td>
<td>0.10 g</td>
<td>Glycogen loss (occasional)</td>
<td>Extensive to massive necrosis</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>0.03 g</td>
<td>Little or no effect</td>
<td>Glycogen loss and minimal necrosis</td>
</tr>
<tr>
<td>1,4-Dichlorobenzene</td>
<td>0.1 g</td>
<td>Little or no effect</td>
<td>Little or no effect</td>
</tr>
<tr>
<td>Benzene</td>
<td>0.25 ml</td>
<td>No specific lesions</td>
<td>No specific lesions</td>
</tr>
</tbody>
</table>

* As described in Methods.
The preferential binding of radioactivity in the necrotic, centrolobular areas shows an association between the binding of the compound and the tissue damage.

**In vitro studies with GSH**

To demonstrate that bromobenzene is converted to an active intermediate which alkylates nucleophilic groups, we incubated [14C]bromobenzene with GSH, an NADPH-generating system, and microsomes and soluble fraction from liver of rats, previously treated with phenobarbital as described in Methods. Paper chromatograms of the incubation mixture revealed a radiolabeled spot, with an Rf value of 0.35, which reacted with ninhydrin (Fig. 3). A peak with the same Rf value was found when [35S]GSH and unlabeled bromobenzene were used instead of [14C]bromobenzene and unlabeled GSH. These results indicate that the peak contained a GSH-bromobenzene conjugate. Omission of the soluble fraction from the system markedly decreased the formation of the [14C]bromobenzene-GSH conjugate (Table 2); this shows that a soluble enzyme was required for maximal formation of the conjugate. Moreover, the conjugate did not form when the microsomes, the NADPH-generating system, or O2 were omitted from the incubation system. When the complete system was incubated in a CO-O2 (9:1) atmosphere, the formation of the conjugate was markedly reduced, which indicates that an NADPH-dependent CO-sensitive enzyme was also required. Furthermore, the alkylation of GSH was considerably decreased when liver microsomes and soluble fraction from rats not previously treated with phenobarbital were used (Table 2). This suggests that the barbiturate treatment increased the activity of the rate-limiting enzyme in the mixture. These findings are consistent with the view that bromobenzene is converted to an active intermediate by a cytochrome P-450 enzyme in liver microsomes and that this intermediate is converted to a GSH conjugate by an enzyme in the soluble fraction.

All the compounds that produced liver necrosis formed conjugates with GSH (Table 3) in the presence, but not in the absence, of liver microsomes from either normal or phenobarbital-treated rats. For example, GSH conjugates were formed with chlorobenzene, iodobenzene, and 1,2- and 1,3-di-
chlorobenzene but not with 1,4-dichlorobenzene. Hexobarbital and ethylmorphine were included as negative controls, because they are metabolized by cytochrome P-450 in liver but are not hepatotoxic. Although naphthalene is not a potent hepatotoxin, it formed considerable amounts of a GSH conjugate.

**DISCUSSION**

A number of chemically inert aromatic hydrocarbons, especially those containing halogen atoms, are converted by hepatic microsomal enzymes to alkylating agents that produce centrolobular necrosis by reacting with tissue macromolecules. For example, the chemically unreactive bromobenzene cannot by itself covalently bind to tissue components, but our results show that this compound in vivo forms a covalent link with liver macromolecules and that the amount of the covalently bound bromobenzene is considerably greater in the necrotic areas than in nonnecrotic areas of liver (Fig. 2).

The important role of microsomal enzymes in the "activation" of aromatic hydrocarbons is emphasized by the following facts: the severity of necrosis is increased in animals that were first pretreated with phenobarbital, a drug which causes a proliferation of the endoplasmic reticulum in centrolobular hepatocytes (8) and induces the synthesis of the enzymes that metabolize foreign compounds (9); the severity of necrosis is decreased (unpublished data) by β-diethylaminoethyl diphenylpropylacetate (SKF 525A), which decreases the activity of these enzymes (10); and the in vitro formation of GSH conjugates of halogenated hydrocarbons requires liver microsomes (7), and is enhanced in microsomes from phenobarbital-treated rats.

Previous studies on the mechanism of mercapturic acid and dihydrodiol formation both in vivo and in liver preparations have led to the view that a number of aromatic hydrocarbons are initially converted to epoxides by liver microsomes (11, 12); indeed, direct proof for the formation of naphthalene epoxide was recently obtained by Jerina et al. (13, 14). According to the current view, the epoxides are then converted nonenzymatically to phenols or enzymatically to their GSH conjugates and dihydrodiol derivatives (15, 16). Subsequently, the GSH conjugates are transformed and excreted in urine as premercapturic acids while the dihydrodiol derivatives are converted to catechols. The reactions with bromobenzene are shown below.
According to our view, the epoxides of bromobenzene and other hepatotoxic aromatic hydrocarbons produce necrosis by alklylation of various macromolecules in hepatocytes. The conversion of the epoxides to phenols, dihydrodiols, and GSH conjugates are alternative pathways that compete with the reaction of the epoxides with tissues. Whether necrosis is produced by a particular aromatic hydrocarbon may depend on its chemical reactivity and on the relative rates at which it is formed and converted to nontoxic metabolites. In some cases the epoxide is formed with difficulty; for example, the GSH conjugate of 1,4-dichlorobenzene is not detectable in vitro (Table 2). Other epoxides may be rapidly converted to nontoxic metabolites; perhaps naphthalene epoxide illustrates this case, since naphthalene is readily converted to its GSH conjugate. Thus, the formation of GSH conjugates and dihydrodiol analogues does not necessarily parallel the incidence of necrosis, but does indicate that the parent compound or one of its metabolites is a potential alkylating agent.

Necrosis may also be caused by active metabolites other than epoxides. For example, CCl₄ causes centrolobular necrosis, presumably by being converted in microsomes to an active intermediate which, in turn, reacts with liver components (17), since prior pretreatment of rats with phenobarbital enhances the toxic effects of CCl₄ (18, 19). Similarly, dialkynitrosamines are thought to exert their hepatotoxic effects through the formation of diazo alkylating derivatives (20).

Some compounds produce necrosis in the perportal rather than in the centrolobular regions of the liver. Reid et al. (21) have confirmed the findings of Eger (22) that allyl alcohol and its precursor, allyl formate (23), produce perportal rather than centrolobular necrosis and have shown that the hepatotoxicity of these compounds is not affected by previous treatment of the rats with phenobarbital. It should be pointed out that these hepatotoxic agents are activated by enzymes other than those in endoplasmic reticulum. In fact, Rees and Tarlow (24) have shown that allyl formate is converted to the highly reactive aldehyde, acrolein, by alcohol dehydrogenase and that this enzyme is localized in the perportal zone.

In conclusion, the liver (and perhaps other organs) can convert stable organic compounds to potent alkylating agents, which form covalent bonds with tissue macromolecules. The possibility must be considered that the therapeutic agents that occasionally cause damage to liver, kidney, bone marrow, and other tissues may also form small amounts of alkylating agents in these organs. Similarly, allergic responses to drugs may be mediated through antigens formed by the reaction of body proteins with trace amounts of chemically active drug metabolites (25). According to this view, the drug-metabolizing enzymes not only "detoxify" drugs but may also form active intermediates that mediate a variety of toxic effects.

We thank Drs. Alan Rabson and Jack Goggins for their helpful advice on the pathological studies and Mrs. Barbara Hodshon, Mrs. Mary Alice Larson, Mr. Nathan Miller, and Mr. John George for expert technical assistance.

These studies were presented in part at the New York Academy of Science Symposium on Drug Metabolism in Man, New York, N.Y., June 21–23, 1970 and at the Fall Meeting of the American Society for Pharmacology and Experimental Therapeutics, Palo Alto, Calif., August 23–27, 1970 (21).