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Biliary excretion of metabolities of bromobenzene

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THIS LABORATORY has previously reported that bromobenzene is converted by liver microsomes to an arene oxide intermediate which binds covalently with protein and perhaps other tissue macromolecules.[1,2] The degree of covalent binding of this intermediate to tissue macromolecules appears to be directly related to the hepatotoxic properties of bromobenzene.[1–4] The arene oxide, however, also reacts with glutathione (GSH) to form a bromobenzene–GSH conjugate,[1,5] which ultimately is converted to bromophenylmercapturic acid, one of the major urinary metabolities of bromobenzene.[6] Since it has been shown that drug conjugates having a molecular weight greater than 300 are often excreted in the bile of rats[7,8] and since the molecular weight of the bromobenzene–GSH conjugate is 480, it seemed possible that this conjugate might be excreted in the bile of rats. The present study not only confirms this view, but also shows that the biliary excretion of the bromobenzene–GSH conjugate is increased by prior treatment of rats with phenobarbital and is decreased by the prior administration of diethylaminoethyl[2,2]–diphenylvalerate HCl (SKF 525-A).

Male Sprague–Dawley rats (Hormone Assay) weighing 200–220 g were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), the bile ducts were cannulated with polyethylene No. 10 tubing, and bile was collected in preweighed vials. A heating lamp was placed over each animal to maintain normal body temperature. After an initial 30-min collection period, ^{14}C-bromobenzene dissolved in rat plasma was administered via the femoral vein at a dose of 20 mg/kg (sp. act., 2 μCi/μmole) and bile was collected in the preweighed vials at intervals up to 3 hr. This dose of bromobenzene was selected because of its limited solubility in rat plasma and because it produced no apparent histological changes in the liver at 3 hr. Three hr after the administration of bromobenzene, the animals were sacrificed and urine was collected from the bladder. Since heptane extraction of the bile or urine removed no radioactivity, it was assumed that no free bromobenzene was excreted in these fluids. Therefore, the total metabolites excreted were determined by liquid scintillation counting of aliquots of bile or urine. Aliquots of bile and urine were chromatographed on Whatman No. 3 MM paper, developed with butanol–acetic acid–water (4:1:2) and then scanned to reveal radiolabeled peaks corresponding to the various metabolites of bromobenzene.

Figure 1 indicates the cumulative excretion of ^{14}C-bromobenzene metabolites appearing in the bile. Each point represents the mean value for five to six animals and is expressed as the per cent of administered ^{14}C-bromobenzene. In these experiments, the effects of pretreatment with phenobarbital (80 mg/kg, i.p., daily for 3 days) or SKF 525-A (75 mg/kg, i.p., 2 hr before bromobenzene) on the biliary excretion of bromobenzene metabolites were also investigated. Within the first 30 min., 11 per cent of the administered ^{14}C-bromobenzene appeared in the bile of control rats as bromobenzene metabolites. Thirty-five per cent was present in the bile of phenobarbital-induced rats and only 3 per cent was present in the bile of rats pretreated with SKF 525-A. During the second 30-min collection period, 18 per cent of the administered dose was excreted in the control and phenobarbital-pretreated

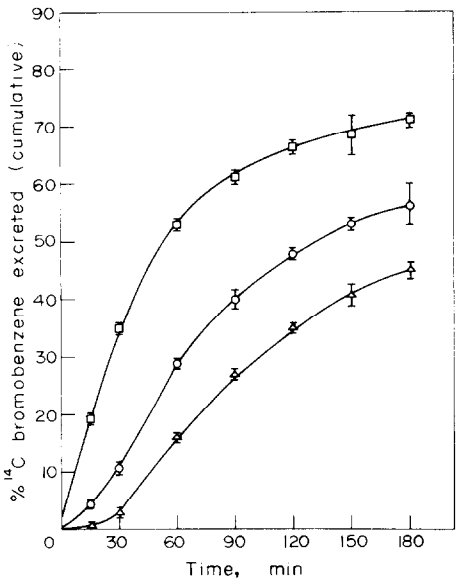

FIG. 1. Cumulative excretion of ^{14}C-bromobenzene metabolites in the bile of rats receiving 130 μmoles/kg of ^{14}C-bromobenzene intravenously (sp. act. 2 μCi/μmole). (○), Non-treated rats; (□) phenobarbital-treated rats (80 mg/kg, i.p. for 3 days); (△), SKF 525-A-treated rats (75 mg/kg, i.p. 2 hr before bromobenzene). Bars represent standard error of the mean.

rats and 15 per cent in the SKF 525-A-pretreated rats. At additional time points, the rate of excretion of metabolites of bromobenzene in the bile declined in all three groups (Table 1). At the end of 3 hr, the cumulative excretion in the bile was 56 per cent in controls, 71 per cent in the phenobarbital-induced rats and 45 per cent in the SKF 525-A-treated animals. The total ^{14}C-metabolites of bromobenzene appearing in the bile and urine at the end of 3 hr was 80 per cent of the administered dose in the untreated controls, 94 per cent in the phenobarbital-pretreated rats and 57 per cent in the SKF 525-A-treated rats.

Pretreatment of rats with phenobarbital produced a statistically significant increase in bile flow, especially during the 0–30 and 30–60 min time intervals (Table 1). Pretreatment with SKF 525-A consistently decreased the bile flow, but the decrease was significant only during the 0–30 min collection period (Table 1). Since studies *in vitro* on the metabolism of bromobenzene have shown that phenobarbital pretreatment results in a 20-fold increase in the metabolism of bromobenzene,[1,5] the increase in biliary excretion of ^{14}C-bromobenzene metabolites probably results from a combination of increased formation of metabolic products and an increase in biliary flow. Similarly, the decreased biliary excretion of metabolites following SKF 525-A pretreatment can result from a decrease in biliary flow, a decrease in the rate of metabolism,[5,*] or a combination of both effects. Klasssen[9] has already shown that phenobarbital markedly increases biliary flow and biliary excretion of metabolites of drugs.

Figure 2a is a representative radiochromatogram tracing of an aliquot of bile obtained from a control rat during the first 30 min of bile collection. Three major metabolites account for approx. 90 per cent of the total radioactivity remaining in this paper. Peak 1 (R_f = 0·56) represents 50 per cent of the radioactivity, while peak 2 (R_f = 0·7) and peak 3 (R_f = 0·75) represent 23 and 17 per cent respectively. At all subsequent times, the biliary excretion of bromobenzene metabolites follows a similar pattern. Pretreatment of rats with phenobarbital or SKF 525-A did not alter the pattern of biliary excretion of bromobenzene metabolites.

When bile was incubated for 16–18 hr with β-glucuronidase or aryl sulfatase and then chromatographed and scanned for radioactivity, the pattern of biliary excretion of the bromobenzene metabolites was unchanged. Hydrolysis of an aliquot of bile with 6 N HCl for 16 hr at 100° resulted in the elimination of peak 1, a significant reduction in peak 2, and an increase in peak 3. Our interpretation of these results is that peak 1 represents the bromobenzene–GSH conjugate as postulated by Brodie *et al.*[1] This metabolite has the same R_f value as the GSH-dependent metabolite of bromobenzene isolated from a system *in vitro*, as described by Sipes and Krishna.[5*] Since acid hydrolysis eliminated this conjugate

* I. G. Sipes and G. Krishna, in preparation.

TABLE 1. EFFECTS OF PRETREATMENT OF RATS WITH PHENOBARBITAL (80 mg/kg, i.p. for 3 days) AND SKF 525-A (75 mg/kg, i.p. 2 hr before) ON THE BILIARY EXCRETION OF BROMOBENZENE METABOLITES AND ON BILE FLOW*

	Pretreatment					
	None		Phenobarbital		SKF 525-A	
Time after bromobenzene (min)	Bromobenzene metabolites (μmoles)	Volume of bile (ml)	Bromobenzene metabolites (μmoles)	Volume of bile (ml)	Bromobenzene metabolites (μmoles)	Volume of bile (ml)
0–30	2·9 ± 0·38	0·59 ± 0·04	9·1 ± 1·1†	1·00 ± 0·06†	0·80 ± 0·10†	0·39 ± 0·02‡
30–60	4·6 ± 0·51	0·55 ± 0·07	4·7 ± 0·56	0·80 ± 0·04‡	3·4 ± 0·30‡	0·42 ± 0·03
60–90	2·9 ± 0·43	0·55 ± 0·06	2·1 ± 0·19	0·70 ± 0·05	2·8 ± 0·42	0·45 ± 0·04
90–120	2·1 ± 0·14	0·47 ± 0·03	1·4 ± 0·08†	0·60 ± 0·04	2·1 ± 0·37	0·42 ± 0·03
120–150	1·3 ± 0·12	0·42 ± 0·04	0·67 ± 0·23†	0·58 ± 0·04	1·6 ± 0·28	0·40 ± 0·04
150–180	0·8 ± 0·05	0·45 ± 0·03	0·76 ± 0·08	0·58 ± 0·06	1·0 ± 0·15	0·46 ± 0·07

* ^{14}C-bromobenzene was administered i.v. at a dose of 20 mg/kg (130 μmoles/kg, sp. act., 2 μCi/μmole).
Each value is the mean of five to six rats ± S.E.
† $P < 0.01$ compared to control rats.
‡ $P < 0.05$ compared to control rats.

peak and reduced peak 2. peak 2 may represent glycine-cysteinyl bromobenzene and peak 3 may represent cysteinyl bromobenzene produced by removal of one and two amino acids from the bromobenzene–GSH conjugate respectively. Peaks 2 and 3 do not represent other known metabolites of bromobenzene such as bromophenol, bromocatechol or bromopheyl dihydrodiol, since the R_f values for these metabolites are higher than those obtained for peaks 2 and 3.

Two major metabolites of bromobenzene appeared in the urine at the end of 3 hr (Fig. 2b). The metabolite with an $R_f = 0{\cdot}68$ represents the glucuronide conjugate of bromophenol, since incubation for 16 hr with β-glucuronidase eliminated this peak.[6] Incubation with aryl sulfatase produced no alteration in the profile of the urinary metabolites. The other major urinary metabolite of bromobenzene is bromophenyl mercapturic acid, represented by the metabolite with $R_f = 0{\cdot}88$. The R_f value of this metabolite corresponds with that of crystalline ^{14}C-bromophenyl mercapturic acid (Fig. 2c) which was isolated from the urine of bromobenzene treated rats and the structure elucidated by mass spectrometry.[6]

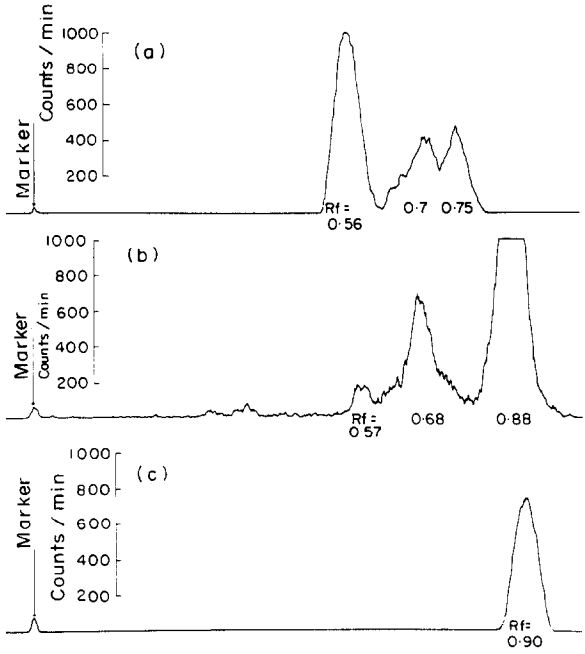

FIG. 2. Representative radiochromatogram of an aliquot of bile (a) and of urine (b) from rats receiving ^{14}C-bromobenzene intravenously. The peak in tracing c is that obtained for crystalline ^{14}C-bromophenyl mercapturic acid isolated from the urine of bromobenzene-treated rats and the structure elucidated by mass spectrometry. Paper chromatograms were developed in a solvent system of butanol acetic acid water (4:1:2).

In conclusion, it is apparent that in the rat the biliary route represents a major means of excretion for certain metabolites of bromobenzene. These biliary metabolites appear to be conjugates derived from the alkylation of GSH by the highly reactive 3,4-bromobenzene arene oxide. This arene oxide is formed by the cytochrome P-450 enzymes of liver microsomes and has been implicated as the agent responsible for the hepatotoxicity produced by bromobenzene.[1]

Since findings by Reid *et al.*[6] and Zampaglione *et al.*[10] indicate that 80 per cent of ^{14}C-bromobenzene administered to control rats can be accounted for in the urine after 24 hr, and we have found that 56 per cent can be accounted for in the bile after 3 hr, the majority of the metabolites excreted in the bile must be reabsorbed from the intestine via the enterohepatic circulation and later excreted by the kidneys.

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Reduction of chloral hydrate to trichloroethanol in brain extracts

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THE PRIMARY product of chloral hydrate metabolism is trichloroethanol[1] which is a more potent hypnotic than chloral hydrate *per se*.[2] The conversion of chloral hydrate to trichloroethanol in the liver has been attributed to the action of alcohol dehydrogenase (EC 1.1.1.1.).[3] Although brain tissue has been shown to be capable of reducing chloral hydrate,[4] the enzymes which catalyze this reaction in brain have not been investigated. Although brain tissue has a small amount of alcohol dehydrogenase similar to the well described liver enzyme,[5] both brain and liver have been shown to contain several reductases capable of metabolizing aldehydes to alcohols.[6–9] In addition, reports[10,11] indicating that the free carbonyl form rather than the hydrated gem diol form of aldehydes was the actual substrate for several dehydrogenases prompted us to investigate the metabolism of chloral hydrate in brain tissue and compare it to the metabolism of chloral and two other aldehydes by liver enzymes. Since differential inhibitors of both alcohol dehydrogenase and aldehyde reductase[12,13] (i.e. pyrazole and barbiturates) are available, we attempted to characterize the chloral hydrate metabolizing activity by the use of inhibitor sensitivities as well as by cofactor and substrate specificities.

Male Sprague–Dawley rats, weighing between 250 and 300 g, were decapitated and brains or livers were quickly removed and homogenized in chilled isolation medium, consisting of 0·32 M sucrose containing 0·05 M sodium phosphate (pH 7·4) and 0·05 mM EDTA. All isolation techniques were performed in the cold (0–4°). Brain homogenates were centrifuged at 27,000 *g* for 30 min and the supernatant fluid was subjected to ammonium sulfate fractionation. Protein precipitating between 0 and 40 per cent saturation (24·3 g/100 ml) with ammonium sulfate and between 40 and 60 per cent saturation (an additional 13·2 g/100 ml) was resuspended in 0·025 M sodium phosphate (pH 7·0) containing 0·5 mM mercaptoethanol and 0·05 mM EDTA. The resuspended fractions and the remaining protein solution were dialyzed for 20 hr against phosphate buffer identical to that used for resuspending the protein. Solutions, after dialysis, were centrifuged at 126,000 *g* for 60 min to remove undissolved proteins and the clear supernatant fluid was used for determination of enzyme activity.

Liver homogenates (10%, v/v) were prepared in an isolation medium consisting of 0·32 M sucrose containing 0·05 mM EDTA and 0·05 M sodium phosphate (pH 7·4), and were centrifuged at 27,000 *g* for 30 min. The supernatant fluid was recentrifuged at 126,000 *g* for 90 min. The clear supernatant fluid obtained from the second centrifugation was used for determination of enzyme activity. Protein concentrations were determined by the method of Lowry *et al.*[14]

Chloral hydrate was twice recrystallized from water. Trichloroethanol was quantified by the method of Cabana and Gessner.[15] This method is based on the formation of a specific chromophore of trichloroethanol. Routinely, the reduction of aldehydes was monitored spectrophotometrically by measuring the decrease in absorbance at 340 nm due to the oxidation of NADH or NADPH. (Assay conditions are