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Inhibition of Rat Liver Cytochrome P-450 by Benzyl Hydrodisulfide

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SUMMARY

The inhibition of rat liver microsomal cytochrome P-450 by benzyl hydrodisulfide has been examined as a model system for the inactivation of cytochrome P-450 seen during the microsomal metabolism of thiono-sulfur-containing compounds. Benzyl hydrodisulfide decreased enzymatic activity toward benzphetamine when incubated with hepatic microsomes prior to the assay for monooxygenase activity. In addition, incubation of microsomes with the hydrodisulfide caused a decrease in the level of cytochrome P-450 detectable as its carbon monoxide complex as well as a decrease in heme detectable as its pyridine-hemochromogen. In a typical experiment, the loss of enzymatic activity, cytochrome P-450, and heme were 65, 56, and 51%, respectively. These data suggest that the major loss of monooxygenase activity and of cytochrome P-450 which was seen on incubation of microsomes with benzyl hydrodisulfide results from an alteration in the structure of the heme moiety of cytochrome P-450. A similar alteration in the heme moiety of cytochrome P-450 is believed to be responsible, in part, for the loss of monooxygenase activity and cytochrome P-450 seen on incubation of parathion and other thiono-sulfur-containing compounds with hepatic microsomes or a reconstituted cytochrome P-450-containing monooxygenase system.

INTRODUCTION

Thiono-sulfur-containing compounds have been shown to have a variety of effects when administered to experimental animals (1–6). A number of these compounds have been shown, both *in vivo* and *in vitro*, to inhibit the hepatic microsomal cytochrome P-450-dependent monooxygenase systems (1–8). The loss of the enzymatic activity is accompanied by a decrease in the level of cytochrome P-450 detectable as its carbon monoxide complex (3).

The cytochrome P-450 monooxygenase-mediated metabolism of the thiono-sulfur-containing compounds, carbon disulfide (5–7) and parathion (8, 9), results in the release of a sulfur atom which becomes covalently bound to microsomal proteins. Covalent binding of the sulfur released during the metabolism of parathion by a reconstituted monooxygenase system as well as hepatic microsomes has been demonstrated to occur almost exclusively to cytochrome P-450 (9). Furthermore, it has been shown that more than one-half of the sulfur covalently bound to cytochrome P-450 can be released upon incubation of the derivatized enzyme with cyanide or dithiothreitol, suggesting the formation of a hydrodisulfide linkage between the sulfur atom released during metabolism and a cysteine group in the cytochrome P-450 polypeptide chain (9, 10). There is also evidence which suggests that a sulfur atom of carbon disulfide, released in the metabolism of this compound by hepatic microsomes, reacts with a cysteine in the polypeptide chain of P-450 forming a hydrodisulfide (7).

Thus, hydrodisulfide linkages formed as a result of covalent binding of the sulfur apparently play an important role in the inactivation of the cytochrome P-450-containing monooxygenase system seen on incubation with thiono-sulfur-containing compounds. No information is currently available concerning whether synthetic hydrodisulfides have similar effects on the cytochrome P-450 monooxygenase systems. This report describes the effect of the synthetic hydrodisulfide, benzyl hydrodisulfide, on rat hepatic cytochrome P-450-containing monooxygenase systems.

MATERIALS AND METHODS

Materials. Benzyl hydrodisulfide was synthesized by the method of Tsurugi and Nakabayashi (11) from acetyl benzyl disulfide prepared by the method of Milligan *et al.* (12). The purity of the hydrodisulfide used in this investigation was ≥95% as judged by iodometric titration. Because of its instability during liquid or gas chromatographic procedures, NMR spectroscopy (13) was used to detect the contamination of benzyl hydrodisulfide with benzyl mercaptan, a contaminant which would lead to overestimation of the hydrodisulfide content as determined by iodometric titration. Also, because of its instability, benzyl hydrosulfide was prepared on the same day it was used in microsomal incubations.

Benzyl mercaptan and dibenzyl disulfide were purchased from Aldrich Chemical Company (Milwaukee, Wisc.) and purified prior to use by distillation and recrystallization from petroleum ether, respectively. Hemin

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chloride, $NADP^+$, and glucose 6-phosphate dehydrogenase were obtained from Sigma Chemical Company (St. Louis, Mo.). Glucose 6-phosphate was obtained from Boehringer Mannheim Corporation (New York, N. Y.). Benzphetamine hydrochloride was a gift of The Upjohn Company (Kalamazoo, Mich.).

Preparation of microsomes. Microsomes were prepared from the livers of phenobarbital-treated male Sprague-Dawley rats weighing 100–150 g. The rats were given phenobarbital (0.1% in the drinking water) for 5 days prior to sacrifice and were killed by decapitation 24 hr after discontinuance of the administration of phenobarbital. The thoracic cavity was opened and the liver was perfused *in situ* with 200 ml of cold 10 mM potassium phosphate buffer (pH 7.4) containing 1.15% KCl and 1 mM EDTA. The microsomal pellet, obtained as described previously (14), was resuspended in isotonic KCl and again centrifuged at 105,000 × *g* for 60 min. This microsomal preparation was free of hemoglobin as judged by difference spectrometry at 420 nm (15).

Incubation conditions. Microsomes were resuspended in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.5) at a protein concentration of 2.3 mg/ml. The microsomal suspension (5 ml) was preincubated at 37° for 5 min followed by the addition of a 0.1-ml aliquot of an ethanol solution of benzyl hydrodisulfide or its breakdown products to give a final concentration of 1 mM. After a 15-min incubation at 37°, the microsomes were isolated by the Ca^{2+} aggregation method (16), washed once with 50 mM potassium phosphate buffer (pH 7.4), and resuspended in the same phosphate buffer. Benzphetamine *N*-demethylation activity, cytochrome P-450, cytochrome b_5, heme, and protein concentrations were then determined using aliquots of this suspension.

Analytical methods. Cytochrome P-450 was quantitated by the method of Omura and Sato (15), heme using the hemochromogen assay (15), and protein by the method of Lowry *et al.* (17).

For determination of benzphetamine *N*-demethylase activity, incubations were carried out according to the method of Hewick and Fouts (18). Formaldehyde formation was determined by the method of Werringloer (19).

For the recording of difference spectra, microsomes were suspended in 50 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol to give a protein concentration of 2.3 mg/ml. The resulting microsomal suspension (6 ml) was divided into two cuvettes and a baseline of equal light absorbance was recorded. Benzyl hydrodisulfide, or its breakdown products, dissolved in ethanol, was added to the sample cuvette. A corresponding amount of ethanol was added to the reference cuvette and the difference spectra were recorded.

RESULTS

When incubated with hepatic microsomes, benzyl hydrodisulfide caused a significant decrease in the magnitude of the 450-nm absorbing peak of the reduced cytochrome P-450-carbon monoxide complex with no concomitant appearance of new peaks in the 350–500 nm region. The decrease in the magnitude of the 450-nm absorbing peak was also dependent upon the concentration of the hydrodisulfide. Neither benzyl mercaptan nor dibenzyl disulfide, known decomposition products of the hydrodisulfide (11, 13), caused any decrease in the magnitude of the reduced cytochrome P-450-carbon monoxide spectra when incubated with hepatic microsomes.

In another experiment, microsomes were reisolated after incubation with benzyl hydrodisulfide, and benzphetamine *N*-demethylase activity was determined. Concentrations of cytochrome P-450, cytochrome b_5, and heme were also determined. As seen in Table 1, incubation of hepatic microsomes with benzyl hydrodisulfide significantly decreased not only the level of cytochrome P-450 detectable as its carbon monoxide complex, but also enzymatic activity toward benzphetamine. The loss of cytochrome P-450, detectable as its carbon monoxide complex, and the loss of enzymatic activity were 56 and 65%, respectively. Similar results were also obtained when the microsomes, after incubation with benzyl hydrodisulfide, were dialyzed for 24 hr against four changes of 50 mM potassium buffer (pH 7.4) containing 20% glycerol and 1 mM EDTA prior to measurement of monooxygenase activity and the concentrations of cytochrome P-450, cytochrome b_5, and heme (data not shown). Thus, it is unlikely that benzyl hydrodisulfide is exerting its inhibitory effect on cytochrome P-450 by coordinating with the heme iron. Incubation of hepatic microsomes with benzyl hydrodisulfide also resulted in a 51% loss of heme detectable as its pyridine-hemochromogen and a 41% loss of cytochrome b_5. On the other hand, incubation of hepatic microsomes with benzyl mercaptan, as described above, did not result in a decrease in the concentration of cytochrome P-450, cytochrome b_5, or heme, or a loss of enzymatic activity (Table 1). In contrast, dibenzyl disulfide caused a decrease in enzymatic activity but no decrease in the concentration of cytochrome P-450, cytochrome b_5, or heme.

These results suggested that one of the effects of interaction of benzyl hydrodisulfide with the microsomal cytochromes, P-450 and b_5, was alteration of the structure of the heme moieties. It has been reported that a trough appears in the 400- to 420-nm region of difference spectra of methemalbumin, hemin chloride, and cytochrome P-450 during NADPH-cytochrome P-450 reductase-mediated heme destruction (20). Incubation of he-

TABLE 1

Effects of benzyl hydrodisulfide and related compounds on cytochrome P-450 and cytochrome b_5

Liver microsomes from rats pretreated with phenobarbital (2.3 mg of protein per milliliter) were incubated with the various substrates (1 mM) at 37° for 15 min. The microsomes were re-isolated as described under Materials and Methods. Benzphetamine *N*-demethylase activity and the levels of cytochrome P-450, cytochrome b_5, and heme were determined also as described under Materials and Methods. All results represent the mean ± standard deviation of triplicate determinations using the same preparation of microsomes.

Substrate[a]	Benzphetamine *N*-demethylase	Cytochrome P-450	Cytochrome b_5	Heme
	nmoles/mg protein/min	*nmoles/mg protein*		
None	12.37 ± 0.71	1.37 ± 0.05	0.34 ± 0.03	1.72 ± 0.09
$PhCH_2SSH$	4.33 ± 0.12	0.60 ± 0.11	0.20 ± 0.02	0.88 ± 0.10
$PhCH_2SH$	11.11 ± 0.81	1.24 ± 0.10	0.32 ± 0.02	1.63 ± 0.06
$(PhCH_2)_2S_2$	7.67 ± 0.62	1.27 ± 0.11	0.33 ± 0.03	1.62 ± 0.12

[a] Ph, phenyl.

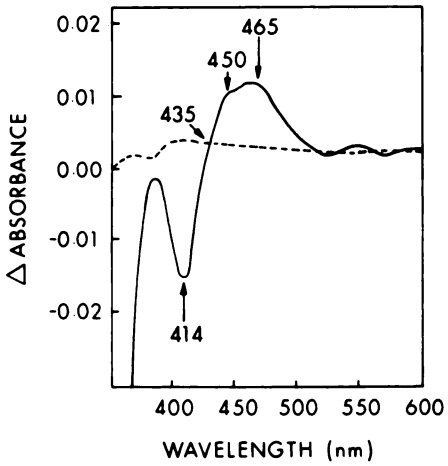


FIG. 1. *Difference spectra induced by benzyl hydrodisulfide*

The experimental procedures are described under Materials and Methods. The *dashed line* represents the baseline obtained in the absence of benzyl hydrodisulfide. The *solid line* indicates the difference spectrum induced by benzyl hydrodisulfide. The concentration of the hydrodisulfide was 0.5 mM.

patic microsomes with benzyl hydrodisulfide resulted in the formation of an unusual difference spectrum with a broad peak centered at 465 nm and a trough at 414 nm (Fig. 1). The magnitude of the difference spectra induced was a function of the concentration of the hydrodisulfide as well as a function of the incubation time. Furthermore, extensive dialysis of the contents of the reference and sample cuvettes following the recording of the difference spectrum caused no change in the difference spectrum induced by the hydrodisulfide. Thus, it is unlikely that the difference spectrum is due to benzyl hydrodisulfide acting as a ligand to the heme iron. As previously reported (21), a characteristic difference spectrum with a peak at 471 nm and a trough at 421 nm was obtained upon addition of an ethanolic solution of benzyl mercaptan to the suspension of hepatic microsomes. Dibenzyl disulfide produced a Type I difference spectrum when added to the liver microsomal suspension.

The ability of benzyl hydrodisulfide to alter the structure of the heme moieties of cytochrome P-450 and b_5 was further examined by incubating benzyl hydrodisulfide with hemin chloride under the same conditions used in the experiments with hepatic microsomes. The results are shown in Table 2. Incubation of hemin chloride with benzyl hydrodisulfide resulted in a 75% decrease in the amount of heme detectable as its pyridine-hemochromogen. Analogous to the results observed using hepatic microsomes, neither benzyl mercaptan nor dibenzyl disulfide caused any decrease in the concentration of heme during the incubation.

TABLE 2

Benzyl hydrodisulfide-mediated alteration in the structure of heme

Each incubation mixture contained hemin chloride (5 μM), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (50 mM, pH 7.5), and substrate (1 mM) in a final volume of 1.5 ml. The reactions were initiated by addition of substrate, and the incubation was carried out at 37° for 15 min in the dark. The heme concentrations were determined at the end of the incubation by the method of Omura and Sato (15). All values are means of triplicate experiments ± standard deviations.

Substrate[a]	Heme
	nmoles/ml
None	4.81 ± 0.45
$PhCH_2SSH$	1.21 ± 0.15
$PhCH_2SH$	4.62 ± 0.36
$(PhCH_2)_2S_2$	4.71 ± 0.31

[a] Ph, phenyl.

DISCUSSION

Incubation of liver microsomes from phenobarbital-pretreated rats with benzyl hydrodisulfide in the absence of NADPH caused a decrease in enzymatic activity toward benzphetamine as well as a decrease in the level of cytochrome P-450 detectable as its carbon monoxide complex. The loss of cytochrome P-450 theoretically accounted for 86% of the loss of enzymatic activity (Table 1). The loss of heme (0.84 nmole) caused by the hydrodisulfide was about 92% of the combined loss of cytochromes P-450 and b_5 (0.91 nmole).

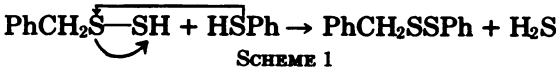
The relationship between the losses of enzymatic activity, cytochrome P-450, and heme seen on incubation of microsomes with benzyl hydrodisulfide in the absence of NADPH is qualitatively similar to that seen on incubation of a purified hepatic cytochrome P-450-containing reconstituted system with the thiono-sulfur-containing compound parathion in the presence of NADPH (10). In the latter case, at least 60% of the loss of enzymatic activity could be attributed to the concomitant loss of heme detectable as its pyridine-hemochromogen. The loss of heme also accounted for approximately 80% of the loss of cytochrome P-450 detectable as its carbon monoxide complex (10). Thus, in both cases, the loss of heme largely accounts for the loss of cytochrome P-450.

An alteration of the structure of the heme moieties of cytochromes P-450 and b_5 is suggested by the benzyl hydrodisulfide-induced appearance of a trough at 414 nm in the difference spectrum of cytochrome P-450 (20) (Fig. 1), the decrease in heme in cytochromes P-450 and b_5 detectable as its pyridine-hemochromogen (Table 1), and the decrease in heme seen on incubation of hemin chloride with benzyl hydrodisulfide (Table 2).

The ability of benzyl hydrodisulfide, in contrast to benzyl mercaptan, to modify the structure of heme is most likely a function of the greater nucleophilicity of the terminal sulfur atom of the hydrodisulfide, as compared with the mercaptan sulfur atom. Hydrodisulfides, in contrast to mercaptans, exhibit the so-called "α-effect" due to the result of strong repulsion between lone-pair electrons of the adjacent sulfur atoms of the hydrodisulfide (22). Because of the strong electron repulsion, hydrodisulfides should exhibit enhanced reactivity with all types of electrophiles, whether saturated or unsaturated (22). For example, xanthine oxidase-catalyzed conversion of xanthine to uric acid and of hypoxanthine to xanthine is believed to be initiated by nucleophilic attack by the hydrodisulfide group at the active site of the enzyme on C-8 of xanthine and C-2 of hypoxanthine, respectively (23).

In incubations of liver microsomes with parathion in the presence of NADPH or with benzyl hydrodisulfide in the absence of NADPH, the loss of heme or cytochrome P-450 is insufficient to account completely for the loss of enzymatic activity. This loss of enzymatic activity in excess of loss of heme or cytochrome P-450 seen on

incubation of liver microsomes with parathion may reflect a selective alteration of the heme of cytochrome P-450 monooxygenases involved in metabolism of benzphetamine or ethoxycoumarin. However, the excess loss of enzyme activity over that of cytochrome P-450 seen on incubation of the microsomes with parathion and benzyl hydrodisulfide may also be the result of structural or conformational changes in the cytochrome P-450 apoprotein resulting from the formation of hydrodisulfide linkages in the polypeptide chain (10). In the case of the excess loss of enzyme activity seen on incubation of liver microsomes with benzyl hydrodisulfide, it is possible that dibenzyl disulfide, a product formed upon decomposition of benzyl hydrodisulfide (11, 13), may have caused a decrease in enzymatic activity without any concomitant loss of cytochrome P-450 (see Table 1). Disulfides are known to react with free sulfhydryl groups in proteins forming mixed disulfides (24). It has been shown that NADPH-cytochrome P-450 reductase (25) and cytochrome P-450 (26) are inhibited by sulfhydryl reagents. Therefore, it is possible that the loss of enzymatic activity seen on incubation of microsomes with dibenzyl disulfide and a portion of the loss of enzyme activity seen on incubation with benzyl hydrodisulfide results from the formation of a mixed disulfide of benzyl mercaptan with cysteine side chains in NADPH-cytochrome P-450 reductase, cytochrome P-450, or both. In the case of benzyl hydrodisulfide the excess loss of enzymatic activity over loss of heme or cytochrome P-450 could also result from the formation of a mixed disulfide as a result of reaction of the sulfhydryl group of a cysteine residue on the internal sulfur atom of the benzyl hydrodisulfide in a manner similar to formation of benzyl phenyl disulfide in the reaction of benzyl hydrodisulfide with thiophenol (13) (Scheme 1, where Ph is phenyl).



SCHEME 1

In conclusion, the synthetic hydrodisulfide, benzyl hydrodisulfide, has been shown to have effects on hepatic cytochrome P-450-containing monooxygenases similar to those seen during the metabolism of thiono-sulfur-containing compounds (1–8). This finding provides support for the hypothesis that hydrodisulfide linkages formed in cytochrome P-450 during the metabolism of thiono-sulfur compounds play an important role in the inactivation of these enzymes. Furthermore, the data suggest that a hydrodisulfide-mediated alteration of the structure of the heme moiety of cytochrome P-450 plays an important role in the inactivation of the enzyme.

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