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Differential Destruction of Cytochrome P-450-Dependent Monooxygenases in Rat and Mouse Kidney following Hexachloro-1:3-butadiene Administration

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SUMMARY

Male rats treated with hexachloro-1:3-butadiene (HCBD) (200–400 mg/kg, i.p.) showed a time-dependent loss of renal cytochrome P-450 which was associated with necrosis in the S_3 segment of the proximal tubule. The maximal effect on cytochrome P-450 was observed 12 hr after HCBD administration (400 mg/kg), when 64% of the renal cytochrome P-450 had been destroyed. Doses higher than 200 mg/kg did not alter the localization of the lesion or the extent of cytochrome P-450 destruction. Associated with this loss was a marked reduction (60–70%) in the cytochrome P-450-mediated metabolism of aldrin. 7-Ethoxycoumarin and *p*-nitroanisole metabolism were also reduced but to a lesser extent (20–30%). However, lauric acid hydroxylation was essentially unchanged. The above data provide evidence for multiple forms of cytochrome P-450 in the kidney and suggest that, in the rat, at least 60% of the total cytochrome P-450 is localized in the S_3 segment of the proximal tubule. Similar losses of renal cytochrome P-450 and its catalytic activity were obtained in female rats treated with HCBD. The administration of HCBD to rats pretreated with the inducer of renal cytochrome P-450 (β-naphthoflavone) resulted in only a 30% loss of cytochrome P-450. Again, only aldrin metabolism was significantly affected, while the induced enzyme activities 7-ethoxycoumarin and 7-ethoxyresorufin remained unaltered. This suggests the presence of at least three separate forms of cytochrome P-450 in the kidneys of β-naphthoflavone-treated rats. It would also appear that the cytochrome P-450 species induced by β-naphthoflavone is either more stable than the majority of the endogenous forms to HCBD-induced inactivation or alternatively is located in a part of the nephron which is not damaged. In mice an almost complete loss of renal cytochrome P-450 was observed 24 hr after HCBD administration (50 mg/kg, i.p.) which was paralleled by a loss of monooxygenase activity. This is associated with the more extensive lesion in this species.

INTRODUCTION

Hepatic cytochrome P-450-dependent monooxygenases play a central role in the metabolism and disposition of lipophilic foreign compounds. Knowledge of the functional role of cytochrome P-450 enzymes present in other organs is still limited. These enzymes are important in the synthesis of androgens in the adrenals (1) and in the metabolism of 25-hydroxy vitamin D_3 in kidney (2). In addition to detoxifying foreign compounds, many extrahepatic cytochrome P-450 enzymes are associated with foreign compound metabolism to toxic products. In the kidney, such metabolism is implicated in the toxicity of paracetamol (acetaminophen), chloroform, methoxyflurane, and several other chemicals (for a review, see ref. 3).

A great deal of progress has been made in the characterization of hepatic cytochrome P-450-dependent monooxygenases (4, 5), and there is now considerable evidence for the existence of multiple forms in control animals. The substrate specificity of these isozymes may be an important factor in determining the metabolism and hence the toxicity of chemicals. Relatively little information is available on the multiplicity of these enzymes in extrahepatic tissues, although multiple forms have been shown to be present in lung [see the review by Philpot and Wolf (6)]. In lung it appears that the distribution of cytochrome P-450 is uneven, the Clara cell and the Type II cell having a higher cytochrome P-450 concentration than other cells (7, 8). The Clara cell has been shown to be more susceptible to damage by the toxins 4-

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ipomeanol and carbon tetrachloride, which require activation via cytochrome P-450 (9).

HCBD[3] is a relatively potent nephrotoxin in rats, mice, and other mammalian species. In rats, the compound produces a very well-defined lesion in the S_3 segment of the proximal tubule (10, 11). In mice, the lesion is more extensive, causing necrosis of both the S_2 and S_3 segments.[4]

In this study we have examined the effects of HCBD on the cytochrome P-450 content and related monooxygenase activities in the kidneys of male and female rats and male mice. These data demonstrate that there are several different forms of cytochrome P-450 in rat kidney and that these cytochromes in this species and the mouse are distributed within specific cell types.

MATERIALS AND METHODS

Chemicals. [1-^{14}C]Lauric acid (28 mCi/mmole, 99% pure) was purchased from the Radiochemical Centre (Amersham, United Kingdom); 12-hydroxylauric acid was purchased from Sigma Chemical Company (London); and 11-hydroxylauric acid was a gift from Dr. T. C. Orton, ICI, Pharmaceuticals Division (Alderley Park, Cheshire). Lauric acid and HCBD were purchased from British Drug Houses (Poole, United Kingdom). Aldrin and dieldrin were purchased from Applied Science Laboratories (Chester). 7-Ethoxyresorufin was synthesized by Dr. S. R. Challend, Wellcome Research Laboratories (Beckenham). All other chemicals were of reagent-grade and of the highest purity commercially available.

Treatment of animals and preparation of microsomes. Mature male and female Wistar-derived (Alderley Park) rats (180–220 g body weight) or male Swiss-derived (Alderly Park) mice (23–28 g body weight) were used. HCBD was administered i.p. at a dose of 200 or 400 mg/kg to rats or 50 mg/kg to mice in corn oil (5 ml/kg). Control animals received corn oil alone (5 ml/kg, i.p.). Following treatment, the animals were fasted for periods of up to 24 hr before use. Animals treated with β-naphthoflavone (BNF) received 100 mg/kg/day i.p. in corn oil for 3 days before administration of HCBD. Control animals for these experiments received BNF but not HCBD. Hepatic and renal microsomal fractions were obtained by differential centrifugation of the tissue homogenates (12). Centrifugation was carried out at 4° in 0.15 M KCl adjusted to pH 7.4 with phosphate buffer. The microsomal pellets were washed once with 0.15 M KCl and then resuspended in 66 mM Tris-HCl buffer (pH 7.4) containing sucrose (0.25 M) and EDTA (5.4 mM). Samples were stored at −20° at a protein concentration of 10–20 mg/ml and used within 2 weeks of preparation.

Enzyme assays and spectral measurements. Cytochrome P-450 concentrations were determined with the method of Omura and Sato (13), using an extinction coefficient of 91 mM^{-1} cm^{-1}. Cytochrome b_5 was measured by the method of Omura and Takesue (14). NADPH-cytochrome *c* reductase activity was measured by the method of Masters *et al.* (15). One unit of activity was determined as the amount of enzyme required to reduce 1 nmole of cytochrome *c* per minute. Hemoprotein concentrations were determined using a Beckman Acta MVI spectrophotometer or a Pye-Unicam SP1800, fitted with a microprocessor to correct the baseline values. Cytochrome P-450 was also measured in control rat renal microsomal fractions (1.6–2.0 mg of protein) incubated with HCBD (1 mM) in 66 mM Tris-HCl buffer (pH 7.4) containing 0.33 mM EDTA (1 ml). The reaction was started by the addition of NADPH (1 mM) and incubated in stoppered tubes at 37° for 90 min. The cytochrome P-450-dependent metabolism of various substrates was determined using the following incubation procedure. Microsomal protein (0.2–2.0 mg) was suspended in 66 mM Tris-HCl buffer (pH 7.4) containing 0.33 mM EDTA (1–2 ml). Substrate dissolved in water, dimethylformamide, or methanol (final concentration 10 μl/ml) was added to give final substrate concentrations as follows: aldrin, 0.2 mM; 7-ethoxycoumarin, 1 mM; 7-ethoxyresorufin, 1 μM; *p*-nitroanisole, 1 mM; and [1-^{14}C]lauric acid, 150 μM and 0.5 μCi. The reactions were started by the addition of NADPH (1 mM). Samples were incubated at 37° for periods of up to 30 min.

For the 7-ethoxycoumarin and *p*-nitroanisole assays the reaction was stopped by the addition of 1 N HCl (0.1 ml). The metabolites 7-hydroxycoumarin and *p*-nitrophenol were then extracted into diethyl ether (3 ml) and then reextracted into 1 M glycine-NaOH buffer (pH 10.5)(2.5 ml). The concentration of 7-hydroxycoumarin was determined fluorimetrically (16), and *p*-nitrophenol was determined from the absorption at 417 nm and from values quantitated using a series of standard solutions. The hydroxylation of lauric acid in positions 11 and 12 was determined by a modification of the method of Orton and Parker (17). After incubation the microsomal protein was precipitated with sulfuric acid (10% w/v). Unchanged substrate and metabolites were extracted into ether (4 ml). The extract was evaporated to dryness and dissolved in methanol (100 μl). An aliquot (50 μl) was injected into a high-pressure liquid chromatography system fitted with a Spherisorb ODS 5 μm column (25 cm × 0.25 cm). The 11- and 12-hydroxy forms of lauric acid were separated by using a methanol/acetic acid/water mixture (57.5:0.5:42.5), and unchanged laurate was eluted with 100% methanol. The retention times of these metabolites were compared with those of authentic standards. The radioactivity in each peak was measured using a Berthold LB 503 MK II flow-through radiochromatographic detector, and peak areas were quantitated with Spectra Physics Model 4100 integrator. Dieldrin, the metabolite of aldrin, was quantified by gas-liquid chromatography according to the method of Wolff *et al.* (18). Where appropriate, 7-ethoxycoumarin and 7-ethoxyresorufin metabolism was assayed by the direct fluorimetric assays reported by Ullrich and Weber (16) and Burke and Mayer (19), respectively. These assays were only used when reaction rates were very fast. The protein content of the microsomes was estimated by the method of Lowry *et al.* (20), using bovine serum albumin as a standard. Plasma urea was measured by the method of Marsh *et al.* (21). Statistical analyses between control and treated means

[3] The abbreviations used are: HCBD, hexachloro-1:3-butadiene, BNF, β-naphthoflavone.

[4] E. A. Lock, I. Pratt, and J. Ishmael, unpublished observations.

were determined by Student's *t*-test, and p values <0.05 considered significant.

RESULTS

Effect of HCBD on rat renal cytochrome P-450 and its monooxygenase activities. Twenty-four hours after administration of a nephrotoxic dose of HCBD (200 mg/kg) to male or female rats, there was a significant reduction in renal cytochrome P-450 concentration (Fig. 1). The reduction in cytochrome P-450 from 0.13 to 0.06 nmole/mg of microsomal protein (54%) was similar in males and females. Higher doses of HCBD (400 mg/kg), which did not alter the pathological localization of the lesion in the kidney (11), did not alter the extent of cytochrome P-450 destruction. The cytochrome P-450 loss could not be accounted for by conversion to the catalytically inactive form, cytochrome P-420. Experiments were also carried out to establish whether a loss of cytochrome could be measured *in vitro* by incubating HCBD (1 mM) with control kidney microsomal fractions fortified with NADPH. No cytochrome destruction was measured. The concentrations of the two other major components of the kidney monooxygenase system, NADPH-cytochrome *c* reductase and cytochrome b_5, remained unaltered by treatment of rats with HCBD (Fig. 1).

A time-dependent loss of renal cytochrome P-450 was observed for the first 12 hr after HCBD administration (Fig. 2). After this period, no further reduction was measured. The initial cytochrome loss was not associated with an increase in plasma urea concentration, used as a measure of renal damage (Fig. 2). However, it did parallel the pathological changes (11). The relationship between the loss of renal cytochrome P-450 and the metabolism of monooxygenase substrates is shown in Table 1. When results are expressed on a milligram per protein basis, the metabolism of aldrin was reduced by 67% in males and by 65% in female samples. A significant, but less marked, reduction in 7-ethoxycoumarin metabolism was observed—32% and 45% in males and females, respectively, The metabolism of *p*-nitroanisole was reduced, but not significantly. In contrast, the metabolism of lauric acid was unaltered. When the results are expressed on a nanomoles of cytochrome P-450 basis, the turnover number for lauric acid increased in the samples from HCBD-treated animals (Table 1). Very high values (between 53 and 85 nmoles) were obtained, suggesting loss of cytochrome P-450s not associated with lauric acid metabolism. The turnover number of the other substrates did

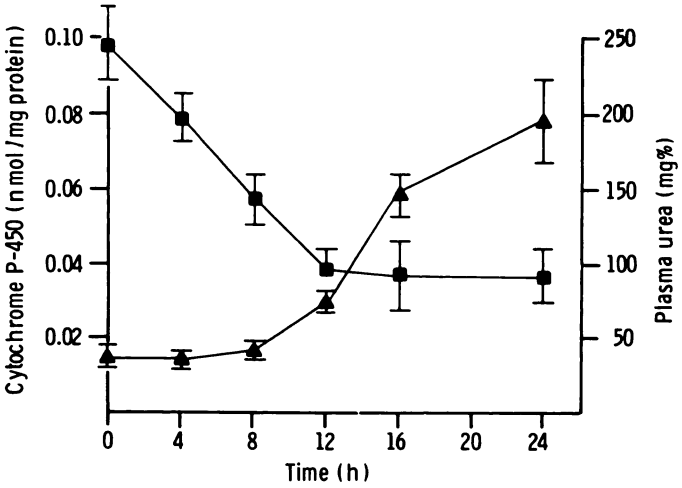

FIG. 2. *Comparison of the rate of HCBD-mediated loss of renal cytochrome P-450* (■) *with changes in plasma urea concentration* (▲)

HCBD (400 mg/kg, i.p.) was administered to male rats, and renal microsomes and blood samples obtained at the times shown. Each point represents the mean ± standard error of four determinations.

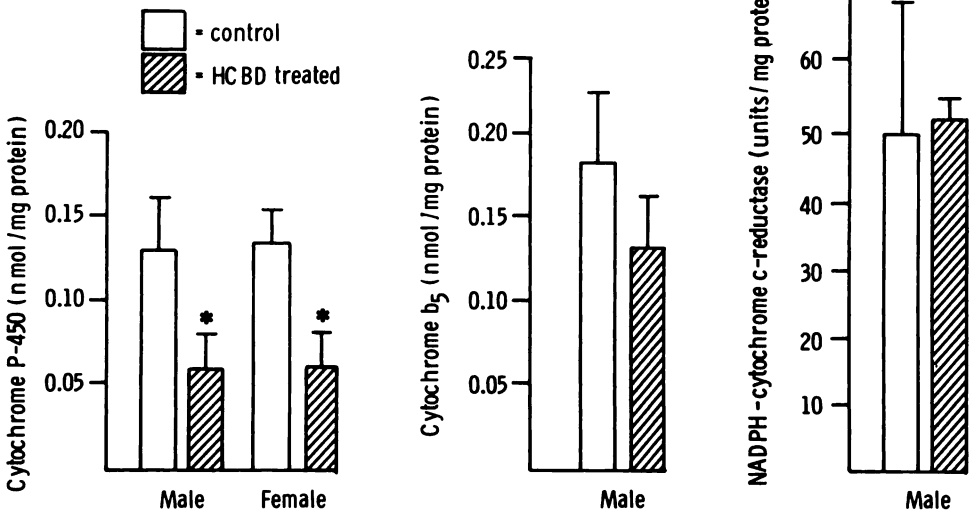

FIG. 1. *Effect of HCBD on renal cytochrome P-450-dependent monooxygenase components in male and female rats*

HCBD (200 mg/kg, i.p.) was administered 24 hr before preparation of renal microsomal fractions. Other details are described under Materials and Methods. Values shown are means ± standard error of at least six determinations; *asterisks* denote statistically significant differences from control values ($p < 0.01$).

not change significantly following HCBD administration, suggesting that the cytochrome loss paralleled the loss of these activities (Table 1).

Effect of HCBD on rat hepatic cytochrome P-450 and its monooxygenase activities. In contrast to the changes observed in the kidney, HCBD did not significantly reduce the hepatic cytochrome P-450 content in the male rat, [control, 0.80 ± 0.11; treated, 1.16 ± 0.32 nmoles/mg of protein (n = 6)]. A slight but significant increase in cytochrome P-450 reductase [from 248 ± 43 to 322 ± 5 units/mg of protein (n = 6)] was measured ($p < 0.01$). Cytochrome b_5 levels were unchanged. In hepatic microsomes from male animals, HCBD treatment did not affect 7-ethoxycoumarin, *p*-nitroanisole, aldrin, or lauric acid metabolism (Table 2). However, the metabolism of 7-ethoxyresorufin was significantly increased. Male rats treated with BNF and then dosed with HCBD showed a small (but not significant) increase in the hepatic microsomal metabolism of aldrin (Table 2). The activities of the other substrates were essentially unaffected by HCBD treatment (Table 2).

Effect of HCBD on rat renal cytochrome P-450 and its monooxygenase activities in BNF-treated animals. BNF treatment increased renal cytochrome P-450 and the monooxygenase activities associated with the *O*-deethylation of 7-ethoxycoumarin and 7-ethoxyresorufin (Table 3). When HCBD was administered to male rats pretreated with BNF, there was a 33% reduction in renal cytochrome P-450. The amount of cytochrome lost was 0.1 nmole/mg of protein, compared with 0.07 nmole/mg of protein in rats not treated with BNF (Table 3; Fig. 1). HCBD did not affect NADPH-cytochrome *c* reductase

TABLE 1

Male and female rat renal microsomes: effect of HCBD treatment on cytochrome P-450-dependent monooxygenase activities

Rats were treated with HCBD (200 mg/kg, i.p.), and renal microsomes prepared after 24 hr.

Substrate[a]	Rate of metabolism[b]							
	Male rats				Female rats			
	Control		HCBD-treated		Control		HCBD-treated	
	A[c]	B[d]	A	B	A	B	A	B
7-Ethoxycoumarin (*O*-deethylation)	0.028 ± 0.005	0.24 ± 0.05	0.019 ± 0.001[e]	0.36 ± 0.07	0.018 ± 0.003	0.14 ± 0.01	0.010 ± 0.002[e]	0.19 ± 0.06
p-Nitroanisole (*O*-demethylation)	0.078 ± 0.019	0.68 ± 0.19	0.047 ± 0.026	0.87 ± 0.42	ND[f]	ND	ND	ND
Aldrin (epoxidation)	0.051 ± 0.019	0.44 ± 0.21	0.017 ± 0.014[e]	0.36 ± 0.26	0.052 ± 0.002	0.40 ± 0.20	0.018 ± 0.006[e]	0.34 ± 0.10
Lauric acid (hydroxylation)								
11-OH	1.59 ± 0.45	11.01 ± 1.44	1.31 ± 0.76	18.47 ± 1.38[e]	ND	ND	ND	ND
12-OH	2.10 ± 0.94	17.1 ± 1.86	1.92 ± 0.77	33.45 ± 7.69[e]	ND	ND	ND	ND
Total	3.69 ± 1.64	28.17 ± 1.87	3.23 ± 1.52	52.92 ± 6.80[e]	6.8 ± 0.4	50.5 ± 7.9	5.0 ± 1.5	85.3 ± 8.0[e]

[a] The procedures used to determine the metabolism of the substrates shown are described under Materials and Methods.
[b] Values are shown as means ± standard deviation with at least four animals per measurement.
[c] A = nanomoles per minute per milligram of protein.
[d] B = nanomoles per minute per nanomole of cytochrome P-450.
[e] Significantly different from control ($p < 0.05$).
[f] Not determined.

TABLE 2

Male rat hepatic microsomes: effect of HCBD on cytochrome P-450-dependent monooxygenase activities in untreated and BNF-treated animals

Untreated animals received HCBD (200 mg/kg, i.p.) 24 hr before use; BNF-treated animals received BNF (100 mg/kg/day, i.p.) for 3 days, and 24 hr after the final dose they received HCBD (200 mg/kg, i.p.).

Substrate[a]	Rate of metabolism[b]							
	Untreated animals				BNF-treated animals			
	Control		HCBD-treated		Control		HCBD-treated	
	A[c]	B[d]	A	B	A	B	A	B
7-Ethoxycoumarin	0.98 ± 0.32	1.09 ± 0.42	0.96 ± 0.15	0.87 ± 0.16	7.2 ± 1.9	5.5 ± 1.8	6.5 ± 0.6	6.1 ± 1.4
p-Nitroanisole	0.47	0.57	0.69	0.93	ND[e]	ND	ND	ND
Aldrin	1.10	1.38	1.36	1.84	0.65 ± 0.34	0.52 ± 0.21	1.59 ± 0.52	1.09 ± 0.44
7-Ethoxyresorufin	0.018 ± 0.006	0.019 ± 0.007	0.038 ± 0.009[f]	0.034 ± 0.006[f]	5.1 ± 0.6	3.7 ± 0.3	4.5 ± 0.5	1.09 ± 0.44
Lauric acid (hydroxylation)								
11-OH	1.80 ± 0.38	2.34 ± 1.16	1.48 ± 0.52	1.68 ± 0.49	1.32 ± 0.27	1.09 ± 0.19	1.25 ± 0.40	0.83 ± 0.23
12-OH	1.61 ± 0.40	1.92 ± 0.97	1.44 ± 0.76	1.96 ± 0.50	1.05 ± 0.25	0.89 ± 0.32	1.19 ± 0.15	0.80 ± 0.19
Total	3.21 ± 0.83	4.26 ± 2.12	2.72 ± 0.60	3.65 ± 0.89	2.37 ± 0.22	1.99 ± 0.44	2.43 ± 0.24	1.64 ± 0.18

[a] The procedures used to determine the metabolism of the substrates shown are described under Materials and Methods.
[b] Values are shown as means ± standard deviation or a mean of two determinations.
[c] A = nanomoles per minute per milligram of protein.
[d] B = nanomoles per minute per nanomole of cytochrome P-450.
[e] Not determined.
[f] Significantly different from control ($p < 0.05$).

TABLE 3

Male rat renal microsomes: effect of HCBD on cytochrome P-450-dependent monooxygenase activities in BNF-treated animals

Animals received BNF (100 mg/kg/day, i.p.) for 3 days. Twenty-four hours after the final dose the animals received HCBD (200 mg/kg, i.p.); control animals received BNF but corn oil instead of HCBD. The values shown were obtained using microsomal samples obtained 24 hr after the dose of HCBD or corn oil.

Substrate[a]	Rate of metabolism[b] of BNF-treated animals			
	Control		HCBD-treated	
	A[c]	B[d]	A	B
Cytochrome P-450[e]	0.34 ± 0.02	—	0.24 ± 0.03[f]	—
NADPH-cytochrome *c* reductase[g]	46.2 ± 3.5	—	52.1 ± 10.8	—
Cytochrome b_5[e]	0.34 ± 0.04	—	0.32 ± 0.01	—
7-Ethoxycoumarin	2.3 ± 0.2	7.7 ± 1.1	3.4 ± 0.2[f]	14.3 ± 0.3[f]
7-Ethoxyresorufin	3.2 ± 0.9	8.1 ± 3.4	4.4 ± 0.6	17.6 ± 0.4[f]
Aldrin	0.08 ± 0.01	0.45 ± 0.10	0.03 ± 0.01[f]	0.15 ± 0.3[f]
Lauric acid				
11-OH	0.92 ± 0.35	4.76 ± 1.22	0.87 ± 0.22	5.78 ± 1.62
12-OH	2.34 ± 0.19	12.53 ± 2.18	1.90 ± 0.35	12.80 ± 2.02
Total	3.26 ± 0.48	17.2 ± 1.9	2.82 ± 0.13	18.6 ± 1.4

[a] The procedures used to determine the metabolism of the substrates shown or cytochrome content are described under Materials and Methods.
[b] Values are shown as means ± standard deviation with at least four animals per measurement.
[c] A = nanomoles per minute per milligrams of protein.
[d] B = nanomoles per minute per nanomole of cytochrome P-450.
[e] Nanomoles per milligram of protein.
[f] Significantly different from control ($p < 0.05$).
[g] Units per milligram of protein.

activity or cytochrome b_5 levels in kidney (Table 3). Aldrin metabolism was significantly reduced in the HCBD-treated animals, whereas the metabolism of 7-ethoxycoumarin and 7-ethoxyresorufin was increased (Table 3). The metabolism of lauric acid was unaltered in HCBD-treated rats (Table 3).

Effect of HCBD on mouse renal cytochrome P-450 and its monooxygenase activities. Mice are more susceptible to HCBD-induced kidney damage than are rats, and in mice severe damage to the S_2 and S_3 segments of the proximal tubule occurs.[4] Concomitant with this effect, an almost complete loss of renal cytochrome P-450 was measured (Table 4), and in some samples no cytochrome P-450 could be detected. In addition, cytochrome b_5 and NADPH-cytochrome *c* reductase levels were significantly reduced (Table 4). HCBD treatment caused a marked reduction in the metabolism of all substrates tested (Table 4). There appeared to be very little metabolism of lauric acid in position 11 in the mouse kidney relative to rats (Table 4). When mice were treated with BNF prior to HCBD, the reduction in the concentration of monooxygenase components was very similar to that in untreated animals (Table 5). In spite of the marked loss of cytochrome P-450, the metabolism of 7-ethoxyresorufin was not altered (Table 5). However, the metabolism of the other substrates was reduced in a manner similar to that in mice not pretreated with BNF (Table 5).

DISCUSSION

The substrate specificities of cytochrome P-450 isozymes in extrahepatic tissues and their localization within these organs is an important factor in the reaction sequence that leads to organ-specific, chemical-induced toxicity. In this respect considerable work has been done in characterizing the pulmonary enzyme systems (6); however, very little is known about the kidney (22) and other organs.

The loss of renal cytochrome P-450 in male and female rats following HCBD treatment was accompanied by a reduction in the rates of metabolism of various cytochrome P-450 substrates. The metabolism of aldrin was reduced more than that of 7-ethoxycoumarin and *p*-nitroanisole, whereas lauric acid was essentially unaffected. This difference provides strong evidence for the presence of multiple forms of renal cytochrome P-450 in untreated rats. The activity of renal cytochrome P-450 toward lauric acid has been known for some time (23); however, the turnover number for the substrate appears to be higher than that originally reported, with a rate of up to 85 nmoles/min/nmole of cytochrome P-450 (Table 1). Okita *et al.* (22) provided evidence that different forms of pig renal cytochrome P-450 are involved in the metabolism of lauric acid at positions 11 and 12. If this is the case in the rat, then these enzymes are either resistant to the effects of HCBD or are localized outside the damaged area of the nephron. The extensive loss of renal cytochrome P-450 in mice following HCBD treatment was accompanied by a small loss of cytochrome b_5 and NADPH-cytochrome *c* reductase activity and a marked reduction in the rates of metabolism of various cytochrome P-450 substrates, including lauric acid. The metabolism of lauric acid in mouse kidney (which has not been reported previously) was interesting in that only a relatively small amount of metabolism occurred in position 11. If there is more than one enzyme involved in the metabolism of lauric acid in mouse kidney, then these enzymes appear to be equally susceptible to destruction, as the ratio of 11-OH to 12-OH metabolites remained constant following cytochrome loss by HCBD, indicating

TABLE 4

Male mouse renal microsomes: effect of HCBD treatment on cytochrome P-450-dependent monooxygenase activities

Mice were treated with HCBD (50 mg/kg, i.p.), and renal microsomes were prepared after 24 hr.

Animals	Electron transport components			Substrate metabolism[a]					
	Cytochrome P-450	NADPH-cytochrome *c* reductase	Cytochrome b_5	Aldrin	7-Ethoxy-coumarin	7-Ethoxy-resorufin	Lauric acid		
							11-OH	12-OH	Total
	nmoles/mg protein	*units/mg protein*	*nmoles/mg protein*	*nmoles/min/mg protein*					
Control	0.182 ± 0.035[b]	110 ± 13.1	0.38 ± 0.01	0.024 ± 0.015	0.032 ± 0.004	ND[c]	0.50 ± 0.10	3.46 ± 0.35	3.90 ± 0.43
HCBD-treated	0.027 ± 0.022[d]	71.7 ± 6.9[d]	0.26 ± 0.02[d]	ND	0.009 ± 0.003[d]	ND	0.12 ± 0.09[d]	0.79 ± 0.22[d]	0.91 ± 0.31[d]
% Control	15	65	70	0	28	—	24	23	23

[a] The procedures used to determine the metabolism of the substrates or cytochrome content are described under Materials and Methods.
[b] Values are shown as means ± standard deviation.
[c] ND = not detectable.
[d] Significantly different from control ($p < 0.05$).

TABLE 5

Male mouse renal microsomes: effect of HCBD on cytochrome P-450-dependent monooxygenase activities in BNF-treated animals

Animals received BNF (100 mg/kg/day, i.p.) for 3 days. Twenty-four hours after the final dose the animals received HCBD (50 mg/kg, i.p.); control animals received BNF but corn oil instead of HCBD. The values shown were obtained using microsomal samples obtained 24 hr after the dose of HCBD or corn oil.

Animals	Electron transport components			Substrate metabolism[a]					
	Cytochrome P-450	NADPH-cytochrome *c* reductase	Cytochrome b_5	7-Ethoxy-coumarin	7-Ethoxy-resorufin	Aldrin	Lauric acid		
							11-OH	12-OH	Total
	nmoles/mg protein	*units/mg protein*	*nmoles/mg protein*	*nmoles/min/mg protein*					
BNF untreated	0.233 ± 0.023[b]	167.6 ± 9.9	0.236 ± 0.029	0.018 ± 0.001	0.036 ± 0.010	0.007 ± 0.000	0.5 ± 0.1	2.7 ± 0.7	3.3 ± 0.7
BNF HCBD-treated	0.023 ± 0.007[c]	139.5 ± 7.0[c]	0.195 ± 0.021	0.012 ± 0.003[c]	0.042 ± 0.008	0.003 ± 0.001[c]	0.2 ± 0.1[c]	0.9 ±0.2[c]	1.1 ± 0.2[c]
% Control	17	83	83	67	117	23	40	33	33

[a] The procedures used to determine the metabolism of the substrates or cytochrome content are described in the Materials and Methods section.
[b] Values are shown as means ± standard deviation.
[c] Significantly different from control ($p < 0.05$).

that the cytochrome responsible for the lauric acid metabolism is possibly located in the S_2 segment of the mouse proximal tubule.

In rats treated with BNF, the HCBD-mediated cytochrome P-450 loss was less marked. The induced cytochromes, as measured by their specific activities toward 7-ethoxycoumarin and 7-ethoxyresorufin, were unchanged. It would therefore appear that the cytochrome P-450 species induced by BNF are either more stable than the majority of the endogenous forms or are localized outside the damaged area of the nephron.

The mechanism of renal cytochrome P-450 loss is not clear. If it were due to the direct attack by a chemically reactive metabolite of HCBD formed by cytochrome P-450 itself (e.g., an HCBD-epoxide), then cytochrome P-450 destruction should have been observed *in vitro* in renal microsomal samples or in liver *in vivo*. A similar argument applies if the cytochrome destruction was due to HCBD-induced lipid peroxidation, a reaction known to result in the destruction of hepatic cytochrome P-450 (24). The role of reactive metabolites in this reaction remains to be determined. Attempts to determine whether the reduction was due to loss of the heme moiety were not successful owing to problems associated with measuring relatively small changes in total microsomal heme content (cytochrome P-450 plus cytochrome b_5), where the over-all heme content is low.

Zenser *et al.* (25) showed that cytochrome P-450 in rabbit kidney was predominantly located in the cortex. In a more refined study, Dees *et al.* (26), using immunofluorescence techniques, have shown that NADPH-cytochrome *c* reductase is localized in all segments of the proximal tubules, although the fluorescence appeared stronger in the S_2 and S_3 segments. In addition, these workers have recently reported that the localization of a specific cytochrome form (isolated from minipigs whose antibody cross-reacts with a form found in rats) was predominantly found in the S_3 segment (27). The fact that HCBD-induced renal damage in the rat is exclusively in the S_3 segment of the proximal tubule suggests that it is this segment which contains a high proportion of the renal cytochrome P-450 (60%; Fig. 1). By analogy, the S_2 and S_3 segments in the mouse appear to contain the majority of the cytochrome P-450. These findings imply that in the rat the S_3 segment and in the mouse the S_2 and S_3 segments may be particularly susceptible to damage by chemicals that require activation through the cytochrome P-450 system. This has already been shown to be the case for acetaminophen (28), chloroform (29), and 4-ipomeanol (30).

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REFERENCES

1. Jefcoate, C. R., R. Hume, and G. S. Boyd. Separation of two forms of cytochrome P-450 from adrenal cortex mitochondria. *F. E. B. S. Lett.* **9**:41–44 (1970).
2. DeLuca, H. F., and H. K. Schnoes. Metabolism and mechanism of action of Vitamin D. *Annu. Rev. Biochem.* **45**:631–666 (1976).
3. Hook, J. B., K. M. McCormack, and W. M. Kluwe. Biochemical mechanisms of nephrotoxicity, in *Reviews in Biochemical Toxicology* (E. Hodgson, J. R. Bend, and R. M. Philpot, eds.), Vol. 1. Elsevier/North-Holland, Amsterdam, 53–78 (1979).
4. Coon, M. J., and A. V. Persson. Microsomal cytochrome P-450 a central catalyst in detoxification reactions, in *Enzymatic Basis of Detoxication* (W. B. Jakoby, ed.), Vol. 1. Academic Press, New York 117–134 (1980).
5. Johnson, E. F. Multiple forms of cytochrome P-450: criteria and significance, in *Reviews of Biochemical Toxicology* (E. Hodgson, J. R. Bend, and R. M. Philpot, eds.), Vol. 1. Elsevier/North-Holland, Amsterdam, 1–26 (1979).
6. Philpot, R. M., and C. R. Wolf. The properties and distribution of enzymes of pulmonary cytochrome P-450-dependent monooxygenase systems, in *Reviews in Biochemical Toxicology* (E. Hodgson, J. R. Bend, and R. M. Philpot, eds.), Vol. 3. Elsevier/North-Holland, Amsterdam, 51–76 (1981).
7. Devereux, T. R., C. Serabjit-Singh, S. R. Slaughter, C. R. Wolf, R. M. Philpot, and J. R. Fouts. Identification of cytochrome P-450 isozymes in nonciliated bronchiolar epithelial (Clara) and alveolar type II cells isolated from rabbit lung. *Exp. Lung Res.* **21**:221–230 (1981).
8. Serabjit-Singh, C. J., C. R. Wolf, R. M. Philpot, and C. G. Plopper. Cytochrome P-450: localisation in rabbit lung. *Science (Wash. D. C.)* **207**:1469–1470 (1979).
9. Boyd, M. R. Biochemical mechanisms in chemical-induced lung injury: roles of metabolic activation. *Crit. Rev. Toxicol.* **7**:103–176 (1980).
10. Lock, E. A., and J. Ishmael. The acute toxic effects of hexachlorobutadiene on the rat kidney. *Arch. Toxicol.* **43**:47–57 (1979).
11. Ishmael, J., I. Pratt, and E. A. Lock. Necrosis of the pars recta (S_3 segment) of the rat kidney produced by hexachloro-1:3-butadiene. *J. Pathol.* **138**:99–113 (1982).
12. Remmer, H., J. B. Schenkman, R. W. Estabrook, H. Sasame, J. Gillette, S. Narasimhulu, D. Y. Cooper, and O. Rosenthal. Drug interaction with hepatic microsomal cytochrome. *Mol. Pharmacol.* **2**:187–190 (1966).
13. Omura, T., and R. Sato. The carbon monoxide-binding pigment of liver microsomes. 1. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239**:2370–2378 (1964).
14. Omura, T., and S. Takesue. A new method for simultaneous purification of cytochrome b_5 and NADPH-cytochrome-*c*-reductase from rat liver microsomes. *J. Biochem. (Tokyo)* **67**:249–257 (1970).
15. Masters, B. S. S., C. H. Williams, and H. Kamin. The preparation and properties of microsomal TPNH-cytochrome-c-reductase from pig liver. *Methods Enzymol.* **10**:565–573 (1967).
16. Ullrich, V., and P. Weber. The O-dealkylation of 7-ethoxycoumarin by liver microsomes—a direct fluorimetric test. *Hoppe-Seyler's Z. Physiol. Chem.* **353**:1171–1177 (1972).
17. Orton, T. C., and G. L. Parker. The effect of hypolipidaemic agents on the hepatic microsomal drug metabolising enzyme system of the rat: induction of cytochrome(s) P-450 with specificity towards terminal hydroxylation of lauric acid. *Drug Metab. Dispos.* **10**:110–115 (1982).
18. Wolff, T., E. Deml, and H. Wanders. Aldrin epoxidation, a highly sensitive indicator specific for cytochrome P-450-dependent monooxygenase activities. *Drug Metab. Dispos.* **7**:301–305 (1979).
19. Burke, M. D., and R. T. Mayer. Ethoxyresorufin: direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metab. Dispos.* **2**:583–588 (1974).
20. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
21. Marsh, W. H., B. Fingerhut, and H. C. Miller. Automated and manual direct methods for the determination of blood urea. *Clin. Chem.* **11**:624–627 (1965).
22. Okita, R. T., L. K. Parkhill, Y. Yasukochi, B. S. S. Masters, A. Theoharides, and D. Kupfer. The ω- and (ω-1) hydroxylase activities of prostaglandins A_1 and E_1 and lauric acid by pig kidney microsomes and a purified kidney cytochrome P-450. *J. Biol. Chem.* **256**:5961–5964 (1981).
23. Ellin, A., S. V. Jakobsson, J. B. Schenkman, and S. Orrenius. Cytochrome P-450K of rat kidney cortex microsomes: its involvement in fatty acid ω and (ω-1)-hydroxylation. *Arch. Biochem. Biophys.* **150**:64–71 (1972).
24. Levin, W., A. Y. H. Lu, M. Jacobson, and R. Kuntzman. Lipid peroxidation and the degradation of cytochrome P-450 heme. *Arch. Biochem. Biophys.* **158**:842–852 (1973).
25. Zenser, T. V., M. B. Mattammal, and B. B. Davis. Differential distribution of the mixed function oxidase activities in rabbit kidney. *J. Pharmacol. Exp. Ther.* **207**:719–725 (1978).
26. Dees, J. H., L. D. Coe, J. Yasukochi, and B. S. S. Masters. Immunofluorescence of NADPH-cytochrome *c* (P-450) reductase in rat and minipig tissues injected with phenobarbital. *Science (Wash. D. C.)* **208**:1473–1475 (1980).
27. Dees, J. H., L. K. Parkhill, R. T. Okita, Y. Yasukochi, and B. S. S. Masters. Localization of NADPH-cytochrome *c* (P-450) reductase and cytochrome P-450 in animal kidneys. *J. Appl. Toxicol.* **1**:xviii (1981).
28. McMurty, R. J., W. R. Snodgrass, and J. R. Mitchell. Renal necrosis, glutathione depletion and covalent binding after acetaminophen. *Toxicol. Appl. Pharmacol.* **46**:87–100 (1978).
29. Ilett, K. F., W. D. Reid, I. G. Sipes, and G. Krishna. Chloroform toxicity in mice: correlation of renal and hepatic necrosis with covalent binding of metabolites to tissue macromolecules. *Exp. Mol. Pathol.* **19**:215–229 (1973).
30. Boyd, M. R., and J. S. Dutcher. Renal toxicity due to reactive metabolites formed *in situ* in the kidney: investigations with 4-ipomeanol in the mouse. *J. Pharmacol. Exp. Ther.* **216**:640–646 (1981).

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