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Cytochrome P-450-dependent metabolism of 1,1,2,2-tetrachloroethane to dichloroacetic acid *in vitro*

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During the past decade, it has become increasingly evident that many of the adverse effects of halogenated hydrocarbons, which include liver and kidney damage [1] and cancer [2], can be attributed to reactive intermediates of the parent compound formed during its metabolism by the cytochrome P-450-dependent monooxygenase system [3]. In recent years, considerable interest has been devoted to elucidating the mechanisms by which chlorinated ethylenes such as vinyl chloride and trichloroethylene are activated to toxic intermediates [4]. The chlorinated ethanes, which are less widely used [1, 5] in part due to higher acute toxicity, have received less attention.

1,1,2,2-Tetrachloroethane (tetrachloroethane) has been shown recently to be a liver carcinogen in mice, whereas the bioassay provided no convincing evidence for carcinogenic potential in rats [6]. Tetrachloroethane is hepatotoxic in several species including man [5], and it is genetically active in three different test systems including the Ames test [7, 8]. The species difference in carcinogenic potential and the lack of direct cytotoxicity of tetrachloroethane toward Ehrlich–Landschütz diploid ascites tumor cells [9] are consistent with a requirement for metabolic activation of the compound. Tetrachloroethane metabolism has been demonstrated *in vitro* by monitoring dechlorination [10] and substrate disappearance [11], but in neither study were any metabolites identified. At present, little information is available on the pathways by which toxic intermediates might be formed.

The major urinary metabolites found upon *in vivo* administration of tetrachloroethane to mice are dichloroacetic acid (DCA), trichloroethanol, and trichloroacetic acid, the latter two apparently formed via oxidation of trichloroethylene, a decomposition product of tetrachloroethane in aqueous media. DCA was postulated to form by oxidation of dichloroacetaldehyde [12]. However, in light of recent results obtained with chloroform [13], dichloromethane [14], and chloramphenicol [15], an equally attractive pathway for the formation of DCA is hydrolysis of dichloroacetyl chloride; this could be formed by a cytochrome P-450-dependent hydroxylation of tetrachloroethane to give $CHCl_2C(OH)Cl_2$, followed by spontaneous dehydrochlorination to give the acyl chloride, $CHCl_2COCl$.

Tetrachloroethane has been shown to give a type I difference spectrum with liver microsomes from phenobarbital-treated rats [16], and phenobarbital is known to stimulate the metabolism of a number of related compounds [13–15]. Therefore, we have examined the metabolism of tetrachloroethane by intact liver microsomes and a reconstituted monooxygenase system from phenobarbital-treated rats.

Table 1. Effect of cytochrome P-450 antiserum on the metabolism of 1,1,2,2-tetrachloroethane and 7-ethoxycoumarin by liver microsomes from phenobarbital-treated rats

Incubation conditions*	Products† [nmoles·(mg protein)$^{-1}$·min^{-1}]	
	Dichloroacetic acid	7-Hydroxycoumarin
Control	2.70 ± 0.23	5.80 ± 0.11
+ Preimmune serum	2.60 ± 0.05	6.47 ± 0.04
+ Immune serum	1.64 ± 0.04	3.14 ± 0.03

* Microsomes (0.5 mg protein) in 1 ml of 0.05 M HEPES buffer (pH 7.4) containing 15 mM $MgCl_2$ and 0.1 mM EDTA were incubated for 30 min at room temperature with 2 mg preimmune or immune serum from rabbits that had been immunized against the major form of cytochrome P-450 from phenobarbital-treated rats [19]. For the assays of dichloroacetic acid formation, the substrate and NADPH-generating system were then added, and incubations were carried out as described in Methods. For the assays of 7-hydroxycoumarin formation, aliquots corresponding to 0.1 nmole cytochrome P-450 were taken and assayed according to the method of Greenlee and Poland [20].

† Each result is the mean ± S.D. of determinations from duplicate incubations. Under the conditions used, both assays were linear with time and protein concentration.

Methods

Preparation of enzymes. NADPH–cytochrome P-450 reductase and the major form of cytochrome P-450 were purified from liver microsomes of phenobarbital-treated rats as described previously [17]. Both enzyme preparations were ≥ 95 per cent pure as judged by sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis. The specific content of the cytochrome P-450 preparation was 15 nmoles/mg protein based on the protein concentration determined by the method of Lowry *et al.* [18], using bovine serum albumin as the standard.

Incubations with tetrachloroethane. Reaction mixtures were incubated for 5 min at 37° using 0.5 mg microsomal protein (1.0 nmole P-450), 0.5 mM NADP, 10 mM glucose-6-phosphate, 15 mM $MgCl_2$, 0.1 mM EDTA, 1 mM tetrachloroethane (added in 10 μl acetone), and 1 unit glucose-6-phosphate dehydrogenase in a volume of 1 ml of 0.05 M 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) buffer, pH 7.5. Conical tubes (15 ml) equipped with teflon-lined screw caps were used. Incubations were terminated by the addition of 5 ml of ice-cold hexane. After vortex mixing and centrifugation, the hexane layer was discarded. Three additional hexane washes were performed, after which 200 μl of 1 N H_2SO_4 was added to the aqueous phase, which was then extracted with three 2-ml portions of ethyl acetate. The combined ethyl acetate extracts were dried under nitrogen and dissolved in 50 μl methanol. Methyl esters were prepared by addition of 50 μl of diazomethane in diethyl ether prepared from *N*-methyl-*N*-nitroso-*N'*-nitroguanidine. After derivatization, the ether was evaporated with a gentle stream of nitrogen and methanol was added to give a final volume of 100 μl. Aliquots were analyzed for DCA methyl ester by gas chromatography on Tenax GC 60/80 mesh, as described in the legend to Fig. 1.

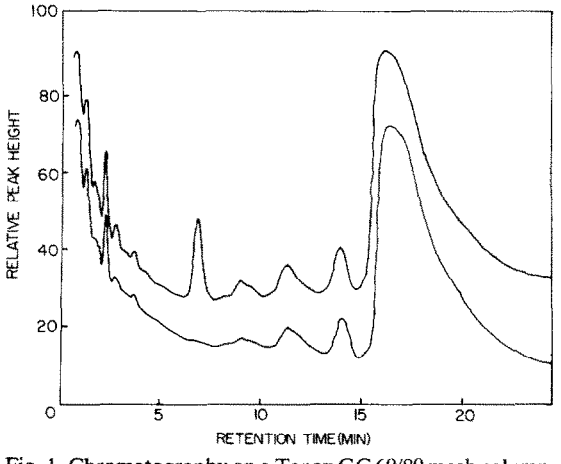

Fig. 1. Chromatography on a Tenax GC 60/80 mesh column (6′ × 2 mm) of diazomethane-treated ethyl acetate extracts from incubations of microsomes or a reconstituted system with tetrachloroethane in the presence (upper curve) or absence (lower curve) of NADPH, as described in Methods. A Varian 1840 gas chromatograph equipped with a Ni^{63} electron capture detector was used. The column was operated at 180° using nitrogen as the carrier gas at a flow rate of 20 ml/min. The injection temperature was 220°, and the detector temperature 300°. The peak at 6.8 mm corresponds to dichloroacetic acid methyl ester.

Incubations of tetrachloroethane with a reconstituted monooxygenase system were carried out essentially as described above except that the microsomes were replaced by 0.45 nmole cytochrome P-450, 1.1 unit reductase, 40 μg dilauryl phosphatidyl choline, and 100 μg sodium deoxycholate; the NADPH-generating system was replaced by 0.4 mM NADPH.

Materials. 1,1,2,2-Tetrachloroethane (min. 98 per cent pure) was purchased from Eastman Kodak Co. (Rochester, NY) and was distilled prior to use. This distillation removed a small amount of lower-boiling impurity, as judged by gas chromatography on Tenax, performed as described in the legend to Fig. 1. The retention time of tetrachloroethane under these conditions was 10.0 min. NADP, NADPH, glucose-6-phosphate, and dilauryl L-3 phosphatidyl choline were purchased from the Sigma Chemical Co. (St. Louis, MO). *N*-Methyl-*N*-nitroso-*N'*-nitroguanidine, 7-ethoxycoumarin, and 7-hydroxycoumarin were from the Aldrich Chemical Co. (Milwaukee, WI). The major β-naphthoflavone-induced form of cytochrome P-450 from rat liver was a gift from Dr. Fred Guengerich. Tenax GC 60/80 mesh was purchased from Applied Science Laboratories, (State College, PA).

Results and Discussion

As seen in Fig. 1, the only significant difference between the gas chromatograms of the diazomethane-treated ethyl

acetate extracts from incubations of microsomes or a reconstituted system with tetrachloroethane in the presence as opposed to the absence of NADPH was the appearance of a peak at 6.8 min, which corresponds to the retention time of standard dichloroacetic acid methyl ester. The identity of the metabolite was confirmed by gas chromatography–mass spectrometry (Fig. 2). The peak at $m/e = 59$ corresponds to the $[CH_3OC{=}O]^+$ ion, and the peaks at 83, 85, and 87 to the $[CHCl_2]^+$ ions.

The recovery of standard dichloroacetic acid from incubation mixtures of microsomes or the reconstituted system lacking NADPH was > 95 per cent, and the method was therefore used to quantify product formation from tetrachloroethane. A reconstituted system containing the major phenobarbital-induced form of cytochrome P-450 catalyzed the formation of 6.3 nmoles DCA·(nmole cytochrome P-450)$^{-1}$·(5 min)$^{-1}$, whereas the major β-naphthoflavone-induced form of the enzyme was only 20 per cent as active. Controls containing the reductase and NADPH but no cytochrome P-450 produced no detectable amount of DCA.

The role of cytochrome P-450 in catalyzing DCA formation by microsomes was assessed using a partially purified antibody against the major phenobarbital-induced form of the enzyme. As shown in Table 1, an amount of antiserum capable of inhibiting deethylation of 7-ethoxycoumarin (a known cytochrome P-450-dependent activity) by 46 per cent inhibited DCA formation by 39 per cent. Higher amounts of serum could not be used due to the formation of emulsions, during the hexane and ethyl acetate extractions, which interfered with the assay. The good agreement between the inhibition of ethoxycoumarin metabolism and of tetrachloroethane metabolism, however, suggests that the majority of the DCA formation in microsomes can be attributed to the cytochrome P-450. When expressed on the basis of the cytochrome P-450 content, DCA formation by microsomes was 6.8 nmoles·(nmole cytochrome P-450)$^{-1}$·(5 min)$^{-1}$, which is comparable to the value of 6.3 obtained with the reconstituted system.

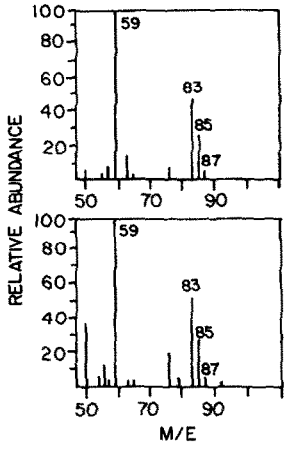

Fig. 2. Gas chromatography–electron ionization mass spectra of authentic dichloroacetic acid methyl ester (upper spectrum) and the methylated metabolite of tetrachloroethane (lower spectrum) isolated as described in Methods. The spectra were recorded on a Ribermag R10-10B instrument equipped with a Serie 31 gas chromatograph and an RDS GC/MS data system. The samples were injected onto a 6′ × 2 mm glass column packed with Tenax GC 60/80 mesh. Helium was employed as the carrier gas at a flow rate of 20 ml/min. The column was operated at 120° for 1 min and then programmed to 200° at 30°/min. The injector temperature was 230°, and the interface temperature 300°. The diverter was opened at 2.0 min and closed at 7.5 min. The mass spectrometer was operated at 70 eV. The retention times of the standard and the methylated metabolite were 6.37 min.

In summary, cytochrome P-450 in both intact liver microsomes and in a reconstituted monooxygenase system was capable of catalyzing the conversion of 1,1,2,2-tetrachloroethane to dichloroacetic acid. The data obtained with the reconstituted system, which lacks aldehyde dehydrogenase, would tend to rule out dichloroacetaldehyde as an obligatory intermediate in DCA formation but would be consistent with an acyl chloride intermediate [13–15].

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