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Characteristics of a Microsomal Dechlorination System

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SUMMARY

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Microsomes were solubilized and the components of the mixed-function oxidase system were isolated. With a reconstituted system containing partially purified cytochrome P-450, NADPH–cytochrome *c* reductase, NADPH, and phospholipid, dechlorination of 1,1,2-trichloroethane was observed. Addition of cytochrome b_5, NADH–cytochrome b_5 reductase, and cyanide-sensitive factor to this system resulted in additional activity, but the cytochrome b_5 system by itself would not dechlorinate. The activity of the complete system was not altered by the presence of the cyanide-sensitive factor. Whole microsomes also were studied and the kinetics of the dechlorination reaction was determined. The K_m was 3.32 mM; V_{max} was 1.09 nmoles of substrate dechlorinated per minute per milligram of protein. The addition of NADH to the NADPH system significantly increased both K_m and V_{max}. The microsomal enzyme system required molecular oxygen; the presence of the cell supernatant fraction produced an increase in the V_{max} of dechlorination.

INTRODUCTION

It has become increasingly evident that dehalogenation reactions play a role in the toxic reactions usually attributed to certain of the parent halogenated compounds, such as the case with carbon tetrachloride (1) and bromotrichloroethane (2). Thus it becomes important to know as many details as possible of the mechanisms of dehalogenation and the enzyme systems responsible for this reaction. Several reports have suggested that there may be more than one mechanism of enzymatic dehalogenation: reductive dehalogenation of carbon tetrachloride to chloroform (3, 4) and of DDT[2] to DDD (5), a glutathione transferase system (6, 7), and oxidative dehalogenation of halomethanes (8) as well as the chlorinated ethanes (9). It remains to be determined which is the most important mechanism in biological systems.

Previously we presented evidence for oxidative dechlorination of the volatile halogenated anesthetics and chlorinated ethanes by a hepatic microsomal enzyme system (9, 10). This reaction depends on NADPH and oxygen, is inhibited by carbon monoxide, and is inducible by phenobarbital but not by methylcholanthrene (11). It has been deduced from these observations that dechlorination is carried out by the hepatic mixed-function oxidase system. Solubilization of hepatic microsomes by the method of Lu *et al.* (12) permits separation of the mixed-function oxidase system and isolation of two fractions: cytochrome P-450 and NADPH–cytochrome *c* reductase. Recombination of these two components plus the addition of lecithin results in an active system for the oxidation of a number of drugs and endogenous

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[2] The abbreviations used are: DDT, dichlorodiphenyltrichloroethane; DDD, dichlorodiphenyldichloroethane.

substrates (13). A report (14) from this laboratory gave evidence that this reconstituted cytochrome P-450 system has the capacity to dechlorinate 1,1,2-trichloroethane.

The presence of cytochrome b_5 in the microsomes, as well as an NADH-dependent cytochrome b_5 reductase, has been known for some time, and it has been suggested that these aid in the function of the microsomal mixed-function oxidase by transferring an electron from NADH to cytochrome P-450 (15). This suggestion has been used to account for the NADH synergism of the NADPH-dependent microsomal oxidation (16) and has been supported more recently by a report by West *et al.* (17), in which they described a cytochrome b_5 shunt to explain this synergism.

Recently another component of the mixed-function oxidase has been isolated, the cyanide-sensitive factor, a lipoprotein that is essential for stearyl coenzyme A desaturation (18) and methyl sterol oxidation (19).

This communication describes the results of studies of the role that these various factors play in the oxidative dechlorination of 1,1,2-trichloroethane.

MATERIALS AND METHODS

Materials. [*U*-^{36}Cl]1,1,2-Trichloroethane (0.05 mCi/mmole) was purchased from New England Nuclear Corporation, diluted with carrier, and used without further purification. Sodium phenobarbital was obtained from Merck & Company. Dilauryl lecithin was purchased from Calbiochem. All other biochemicals and column packings were obtained from Sigma Chemical Company.

Animal treatment. Male Sprague-Dawley rats (300–350 g) were treated for 3 days by addition of 0.2% sodium phenobarbital to their drinking water; then they were fasted overnight and killed (20). Lung and kidney tissues were homogenized with a Teflon pestle as described by Hook *et al.* (21). Liver microsomes were prepared as previously described (10) and stored as a suspension in 0.05 M Tris, pH 7.4, under N_2 at −76° until used. The 105,000 × *g* supernatant (approximately 20 mg of protein per milliliter) was prepared fresh as needed from similarly treated rats.

Preparation of components. Cytochrome P-450 and NADPH–cytochrome *c* reductase was prepared, assayed, and stored according to the method of Lu *et al.* (12). Specific activities were: cytochrome P-450, 3 nmoles/mg of protein; NADPH–cytochrome *c* reductase, 300–400 units/mg of protein.

Microsomal lipid fractions were isolated and prepared from phenobarbital- or methylcholanthrene-treated rats by the procedure of Lu *et al.* (12). Synthetic dilauryl lecithin was used in place of the rat hepatic microsomal lipid fractions in most of the incubations (22).

Detergent-solubilized NADH–cytochrome b_5 reductase and cytochrome b_5 were isolated and purified by the methods of Spatz and Strittmatter (23). The reductase showed no NADPH–cytochrome *c* reductase activity and only reduce $Fe(CN)_6^{3-}$ with NADH, and the cytochrome b_5 contained no cytochrome P-450. The cyanide-sensitive factor was isolated by detergent solubilization, ammonium sulfate fractionation, and column chromatography (18). Specific activities were as follows: cytochrome b_5, 11 nmoles/mg of protein; NADH–cytochrome b_5 reductase, 11.8 μmoles of $Fe(CN)_6^{3-}$ reduced per minute per milligram of protein (11.8 units/mg of protein); cyanide-sensitive factor, 660–720 μg of protein per milliliter.

Incubation mixtures. The incubation mixtures contained an NADPH-generating system ($MgCl_2$, 2.4 μmoles; NADP, 0.5 μmole; glucose 6-phosphate, 1 μmole; and glucose 6-phosphate dehydrogenase, 0.5 unit) and microsomes or isolated microsomal components in a total volume of 2 ml of 0.05 M Tris-HCl buffer, pH 7.4. In most incubations 1 mg of microsomal protein was present.

Microsomal component concentrations used in the assays were as follows: cytochrome P-450 system, 1–2 nmoles of cytochrome P-450, 55–60 units of NADPH–cytochrome *c* reductase, and 100 μg of syn-

thetic dilauryl lecithin; cytochrome b_5 system, 0.6 nmole of cytochrome b_5, 3.4 units of NADH–cytochrome b_5 reductase, and 105 μg of cyanide-sensitive factor protein.

NADH, if added to NADPH or used in place of it, was added as a solution just prior to the addition of the [^{36}Cl]trichloroethane. When indicated, 2 mg of 105,000 $\times$ g supernatant protein were present.

The flasks containing the reaction mixture were flushed for 5 sec with 100% O_2 (5 liters/min) prior to the addition of 1 μl (10.8 μmoles) of [U-^{36}Cl]1,1,2-trichloroethane to start the reaction. In some experiments carbon monoxide or N_2 was bubbled into the incubation mixture for 10–15 min in place of the O_2. The flasks were capped and incubated at 37° for 30 min. To stop the reaction, 0.2 ml of 15% trichloracetic acid was added, and the flasks were allowed to stand uncapped overnight in a hood to allow the excess trichloroethane to volatilize.

In the kinetic studies 0.05 M Tris buffer, pH 7.4, saturated with [^{36}Cl]1,1,2-trichloroethane was added at 0.5–5.5 mM final concentration.

Assays. The total nonvolatile, water-soluble ^{36}Cl-labeled metabolites were determined as previously described (14). The authenticity of the assay was frequently validated by simultaneously performing the more specific inorganic ^{36}Cl assay of Van Dyke and Chenoweth (9).

Protein was determined by the method of Lowry *et al.* (24). Prior to use, the reconstituted cytochrome P-450 system and the reconstituted cytochrome b_5 system were determined to be enzymatically active with the substrates aminopyrine [formaldehyde assay procedure of Nash (25)] and [^{14}C]stearyl coenzyme A [procedure of Oshino *et al.* (26)], respectively.

Other generating systems for incubation mixtures. The superoxide-generating system of Strobel and Coon (27) with xanthine and xanthine oxidase or the peroxide-generating system of Mezey *et al.* (28) with hypoxanthine and xanthine oxidase was used to replace the nucleotide-generating system or the nucleotide as reducing source. Both generating systems were checked for activity by the procedures of Plesner and Kalckar (29).

RESULTS

Dechlorination activity of microsomal fractions from various tissues. Liver, lung, and kidney microsomes exhibited dechlorination activity (Table 1), with the liver by far the most active. The dechlorination activity was stimulated in all cases by the presence of the 105,000 $\times$ g supernatant fraction from the same tissue. The addition of 105,000 $\times$ g supernatant fraction from liver further stimulated the enzymatic activity of the microsomes from all tissues. The 105,000 $\times$ g supernatant fractions alone were not enzymatically active for dechlorination (see Table 3).

Because the liver microsomes had the greatest activity for dechlorination, they were the only microsomes used in the following studies.

Factors affecting microsomal dechlorination. Previous work (9, 10) showed that the dechlorination of trichloroethane by microsomes occurs in the presence of O_2 and is inhibited by carbon monoxide. In incubations performed under N_2 atmosphere, glucose and glucose oxidase were added to the incubation and incubated for 5 min at 37° prior to addition of the trichloroethane, to lower oxygen tension further.

TABLE 1

Dechlorination of 1,1,2-trichloroethane by microsomal fractions of various organs

Microsome source	Total ^{36}Cl-labeled metabolites produced[a]		
	No addition	Supernatant from same tissue[b]	Supernatant from liver[b]
	cpm/mg microsomal protein		
Liver	249 ± 4	382 ± 56	382 ± 56
Lung	60 ± 9	75 ± 13	162 ± 8
Kidney	36	88	194

[a] For liver and lung, values are means ± standard errors for three experiments in duplicate for 30 min of incubation.

[b] The 105,000 $\times$ g supernatant fraction (2 mg of protein).

The results shown in Table 2 indicate that the removal of O_2 greatly inhibited the dechlorination. The supernatant fraction was not found to dechlorinate by itself under oxygen or nitrogen. In addition, it only stimulated dechlorination in an oxygen atmosphere. It should be noted that the reduction of oxygen tension did not completely stop dechlorination.

To study the possible involvement of the system containing NADH–cytochrome b_5 reductase, cytochrome b_5, and cyanide-sensitive factor in dechlorination, the assay was performed with hepatic microsomes in the presence of cyanide ion (an inhibitor of the cyanide-sensitive factor) and stearyl coenzyme A (the substrate for the cyanide-sensitve factor) with and without the addition of NADH, NADPH, and the 105,000 × g supernatant. The addition of cyanide or stearyl-CoA to the incubation mixture did not alter the dechlorination activity.

EDTA, commonly included in microsomal incubations to inhibit lipid peroxidation (30), was found to stimulate microsomal dechlorination (Table 2).

Kinetics of dechlorination with microsomes. The kinetic characteristics of the system (Table 3) show that the presence of the 105,000 × g supernatant had no effect on the K_m but caused an increase in the maximal rate of dechlorination. These effects on microsomal dechlorination were not the result of solubilization of the substrate by the 105,000 × g supernatant.

The addition of NADH also stimulated the dechlorination activity of microsomes in the presence of NADPH. However, the K_m and V_{max} were much lower when NADH was the sole source of electrons than when NADPH was used. The data suggest that the K_m for the NADPH plus NADH system represents the sum of the K_m for the NADPH system and the K_m for the NADH system. The V_{max} for the combined system is higher than the sum of the values for the separate systems. Thus the NADH effect is believed to be synergistic and not facilitative (15–17).

Factors affecting dechlorination with reconstituted microsomal system. Some oxygen seems mandatory for the dechlorination of trichloroethane, although it appears that very little is necessary. Thorough flushing of the system with either carbon monoxide or N_2 greatly inhibited the dechlorination process but did not stop it completely; carbon monoxide was more potent than N_2, with an average inhibition of 73% (Table 4). This may also reflect the difficulty in removing all the oxygen by merely flushing the flasks with another

TABLE 2

Factors affecting microsomal dechlorination

The NADPH-generating system is described in the text. The 105,000 × g supernatant contained 2 mg of protein. Glucose (2 mM) plus glucose oxidase (2.4 units) were added as shown. Relative dechlorination is based on microsomes plus NADPH in O_2 = 100% (19.5 nmoles of trichloroethane consumed in 30 min). Values are means ± standard errors for 4–12 determinations.

Additions to microsomes	Relative dechlorination	
	O_2	N_2
	%	%
NADPH-generating system	100[a]	58 ± 7[b]
NADPH-generating system; glucose + glucose oxidase		26 ± 1[b]
NADH, 0.5 mM	47 ± 4[a, b]	
NADPH-generating system + NADH, 0.5 mM	185 ± 31[a, b]	
NADPH-generating system + 105,000 × g supernatant	166 ± 7[a, b]	61 ± 9[b]
NADPH-generating system + stearyl-CoA, 20 μM	96 ± 9[a]	
NADPH-generating system + EDTA, 10 μmoles	151 ± 21[c]	

[a] Not significantly altered when incubation was performed in the presence of 0.1 mM sodium cyanide.

[b] For difference from microsomes plus NADPH-generating system in O_2, $p < 0.01$.

[c] For difference from microsomes plus NADPH-generating system in O_2, $p < 0.05$.

TABLE 3

Kinetics of dechlorination with microsomes

Values are means ± standard errors for seven experiments performed in duplicate.

Additions to microsomes	K_m	V_{max}[a]
	mM	
NADPH-generating system	3.32 ± 0.62	1.09 ± 0.19
NADPH-generating system + 105,000 × *g* supernatant	3.33 ± 0.71	2.26 ± 0.47
NADPH-generating system + NADH, 1.25–2.50 mM	5.87 ± 0.68[b]	2.63 ± 0.64[b]
NADH, 1.25–2.50 mM	1.80 ± 0.43[c]	0.27 ± 0.11[b]
NADPH-generating system + NADH (1.25–2.50 mM) + 105,000 × *g* supernatant	5.52 ± 1.08[b]	3.56 ± 0.36[b]

[a] In nanomoles of substrate changed per minute per milligram of microsomal protein.

[b] For difference from NADPH-generating system, $p < 0.05$.

[c] For difference from NADPH-generating system, $p < 0.10$.

gas. However, this suggests again the involvement of cytochrome P-450 in the dechlorination reactions.

The addition of N_2 in place of O_2 caused 40–50% inhibition of dechlorination. With intact microsomes, dechlorination was inhibited even more under an N_2 atmosphere with glucose and glucose oxidase present to decrease the oxygen tension further.

The NADPH-generating system was found to be by far the most effective generating system for microsomal dechlorination under aerobic conditions (Table 4). The replacement of either the reductase or reductase and lipid components by a superoxide-generating system or a peroxide-generating system resulted in little or no dechlorination activity. Likewise, when superoxide dismutase was added with the NADPH-generating system and reductase components, no effect on the enzymatic activity was noted.

Phospholipids extracted from livers of phenobarbital- and methylcholanthrene-induced animals were compared with the synthetic phospholipid dilauryl lecithin (Table 4). The phospholipids were quantified on the basis of lipid phosphate, and while optimal enzymatic activity was found with 80 μg of lipid phosphate of lipids extracted from liver microsomes from phenobarbital-induced animals and 150 μg of lipid phosphate of lipids extracted from microsomes from methylcholanthrene-induced animals, neither matched the activity produced by the phospholipid dilauryl lecithin. Inhibition of the dechlorination reaction occurred when more than the optimal amount of each lipid extract was added.

Effect of combined reconstituted P-450 and cytochrome b_5 systems on dechlorination activity. The addition of the components of the cytochrome b_5 system significantly stimulated the reconstituted cytochrome P-450 system in its dechlorination activity; the cytochrome b_5 system alone was not active in dechlorination (Table 5). This stimulation probably resulted from either additional or more efficient flow of reducing equivalents to the cytochrome P-450. NADH was equal to NADPH in driving the dechlorination reaction only if both the reconstituted cytochrome P-450 and cytochrome b_5 systems were present. Thus a network of electron flow pathways is suspected to be present, as has been shown by other observers (15, 31).

DISCUSSION

Previous reports (9, 10) from this laboratory presented evidence that the dechlorination of chloroethanes is carried out by a microsomal system that apparently is similar to a system that oxidizes a number of substrates. In addition, a reconstituted cytochrome P-450 system consisting of three fractions (cytochrome P-450, NADPH–cytochrome *c* reductase, and a phospholipid) was found to carry out dechlorination. The present study extends these findings by further characterizing the microsomal de-

TABLE 4

Factors affecting dechlorination with reconstituted microsomal system

See MATERIALS AND METHODS for components of generating systems. Relative dechlorination is based on the complete P-450 system (with dilauryl lecithin) plus NADPH-generating system = 100% (0.39 ± 0.6 nmole of substrate changed per minute). Values are means ± standard errors for two or three experiments in duplicate.

System	Cytochrome P-450 (1 nmole)	NADPH–cytochrome *c* reductase (55 units)	Dilauryl lecithin (100 μg)	Generating system	Atmosphere	Relative dechlorination
						%
1	+	+	+	NADPH	O_2	100
2	+	+	+	NADPH	N_2	58 ± 5[a]
3	+	+	+	NADPH	CO	27 ± 5[a]
4	+		+	Superoxide	O_2	8
5	+		+	Peroxide	O_2	2
6	+	+	+	NADPH + SOD[b]	O_2	95
7	+	+	PB[c]	NADPH	O_2	80 ± 11[d]
8	+	+	3-MC[c]	NADPH	O_2	56 ± 4[a]

[a] For difference from system 1, $p < 0.001$.

[b] SOD, superoxide dismutase (400 μg of bovine erythrocuprein).

[c] Lipid isolated from rats treated with phenobarbital (PB) or 3-methylcholanthrene (3-MC) by the method of Lu *et al.* (12). Optimum quantities were 80 and 150 μg of phospholipid from the phenobarbital-treated and 3-methylcholanthrene-treated sources, respectively.

[d] For difference from system 1, $p < 0.05$.

TABLE 5

Dechlorination activity of reconstituted cytochrome P-450 and reconstituted cytochrome b_5 systems alone and in combination

See MATERIALS AND METHODS for compositions of reconstituted systems. Relative dechlorination is based on the complete cytochrome P-450 system plus NADPH-generating system = 100% (0.39 ± 0.6 nmole of substrate changed per minute). Values are means ± standard errors for three experiments performed in duplicate.

Additions[a]	Relative dechlorination activity		
	P-450 system	b_5 system	Both systems
	%	%	%
NADPH-generating system	100	9 ± 3[a]	122 ± 6[a]
NADH, 2.7 mM	53 ± 6[a]	7 ± 2[a]	101 ± 7
NADPH-generating system + NADH, 2.7 mM	115 ± 3[b]	8 ± 2[a]	120 ± 11[b]
NADPH-generating system + NADH (2.7 mM) + CN^- (4 mM)	100 ± 4	11 ± 4[a]	125 ± 10[b]

[a] For difference from cytochrome P-450 system plus NADPH-generating system, $p < 0.01$.

[b] For difference from cytochrome P-450 system plus NADPH-generating system, $p < 0.05$.

chlorinating enzyme system and investigating the components necessary for optimal dechlorination activity.

Experiments with the reconstituted system confirmed that the cytochrome b_5 system alone (NADH, NADH–cytochrome b_5 reductase, and cytochrome b_5) does not carry out the dechlorination. However, dechlorination did proceed with the cytochrome P-450 system. Also, when the cytochrome b_5 system was added to the cytochrome P-450 system, the dechlorination activity was slightly enhanced, as seen with other substrates (32), suggesting addi-

tional or more efficient flow of reducing equivalents or that cytochrome b_5 is capable of transferring reducing equivalents from NADH to cytochrome P-450 after the latter has been reduced by NADPH, as was suggested by Hildebrandt and Estabrook (15). An alternative explanation, proposed by Lu *et al.* (17, 33), is that NADPH reduces the cytochrome b_5 system and that the NADH conserves the NADPH by reducing the cytochrome b_5 system. The presence of cyanide ion did not inhibit the enhancement caused by the cytochrome b_5 system, thus indicating that the stimulation by the cytochrome b_5 system must occur prior to the cyanide-sensitive factor in the electron transfer system (Table 5). The addition of NADH and NADPH to intact microsomes (Table 2) resulted in an increase in activity over that with NADPH alone, and the increase was greater than that noted in the reconstituted system (Table 5), perhaps suggesting that the NADPH synergism requires a particular and fixed arrangement of the components to obtain a maximal effect. The NADH synergism also resulted in an increase of both K_m and K_{max} of the dechlorination reaction.

Additional facilitative activity was found when a small amount of the cytosol fraction was added to either the reconstituted system or whole microsomes; the V_{max} was increased but the K_m was unchanged. The active component of the cytosol fraction, presently under investigation, is an extremely labile material and has been partially isolated by column chromatography (34). Two active fractions have been isolated: one of very large molecular weight of approximately 100,000, and another of approximately 10,000. There are three possibilities for its function: (a) the stimulation by the cytosol fraction may be the result of oxidation of an intermediate produced by the microsomes, because it stimulates only in an oxygen atmosphere; (b) the cytosol fraction alters the activity of the enzymes in the microsomes under aerobic conditions; or (c) the cytosol fraction protects the cytochrome P-450 system against peroxidation and thus has EDTA-like activity, as found by Kamataki *et al.* (30) for ethylmorphine dealkylation. Preliminary evidence suggests that the cytosol fraction which stimulates dechlorination has activity in addition to the EDTA-like activity.

It is appropriate at this time that the mechanism of dechlorination be examined. Through a study of a series of chlorinated ethanes it was found that optimal enzymatic dechlorination occurred when there were two halogens and one hydrogen on a terminal carbon (10). Because 1,1,2-trichloroethane has this configuration, it is an excellent substrate for this reaction. Loew *et al.* (35) subjected this same series of chlorinated ethanes to molecular orbital calculations and found that the enzymatic dechlorination correlated with an electron deficiency in one of the orbitals of the carbon carrying the chlorines. The configuration that had the greatest electron deficiency was the optimal configuration for enzymatic dechlorination (10). Thus the chlorinated compounds that are most readily dechlorinated are the ones able to take up an electron most readily. Therefore it is appropriate to consider a nucleophilic or anionic attack on the carbon atom carrying the chlorine, which, as pointed out by Loew *et al.* (35), weakens the C—Cl bond. The nature of the anionic attacking group in this mixed-function oxidation is not known presently, although it presumably is oxygen in the form of a hydroxyl or superoxide radical; either of these could be produced by cytochrome P-450.

Strobel and Coon (27) reported that ω-hydroxylation occurring in a reconstituted cytochrome P-450 system is supported by a superoxide-generating system and is inhibited by superoxide dismutase. However, dechlorination by the reconstituted system as described in this communication is not supported by a xanthine oxidase superoxide-generating system and is not inhibited by superoxide dismutase. A peroxide-generating system does not support the dechlorination. In addition, Levin *et al.* (36) have recently shown that superoxide is not involved in the metabolism of several substrates by the reconstituted systems, which leaves considerable doubt as to the nature of the anionic group responsible for the attack on the chlorinated ethanes and other substrates.

There is no doubt, however, that the data do support an oxidative dehalogenation, although it may not be the only mechanism to consider. In contrast to an oxidative mechanism of dechlorination of chlorinated compounds, a reductive dechlorination has been suggested in several reports. Butler (3) reported a reductive dechlorination of carbon tetrachloride, and this has been supported by more recent investigations (37). DDT has been shown to undergo reductive dechlorination to DDD (5). More recent work in this laboratory indicates that halothane (1,1,1-trifluoro-2-bromo-2-chloroethane), which is debrominated and dechlorinated aerobically, undergoes activation anaerobically by reduction of the bromochloromethane portion of the molecule (37, 38). What role this reduction plays in the dehalogenation and eventual oxidation of this halogenated compound is not known at present. It is possible that halogenated compounds undergo a reduction prior to oxidation, a concerted reaction aerobically but noticeable only under low oxygen tensions. Furthermore, because of the electrophilic nature of halogenated compounds, some consideration should be given to the possibility that reduction is a part of the mechanisms of oxidative dehalogenation. This possibility is currently under investigation.

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