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ENZYMATIC DECHLORINATION

DECHLORINATION OF CHLOROETHANES AND PROPANES *IN VITRO**

RUSSELL A. VAN DYKE† and CATHERINE G. WINEMAN‡

Biochemical Research Laboratory, The Dow Chemical Company, Midland, Mich. 48640, U.S.A.

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Abstract—The enzymatic dechlorination of a series of chloroethanes and chloropropanes was investigated. It was found that these materials were dechlorinated enzymatically by an enzyme system located in hepatic microsomes. This system requires NADPH and oxygen, and is inducible by phenobarbital and benzpyrene, but not by methylcholanthrene. The pH optimum of this system was found to be 8·2. Evidence is presented that a factor is present in the 105,000 *g* supernatant which is necessary for optimum activity. This supernatant factor is not inducible. The dechlorination varied depending on the extent of chlorination of the ethane or propane.

RECENT studies have been directed at elucidating the enzymatic system responsible for dehalogenation by rat hepatic tissue. It has been known since the pioneering work of Heppel and Porterfield[1] that mammals have an enzyme system capable of cleaving the carbon–halogen bond. However, a study of this system has not been undertaken until recent years.

Previous reports from this laboratory have supplied data indicating that an enzyme system is present in rat hepatic microsomes which is capable of cleaving the carbon–halogen bond.[2,3] Furthermore, this enzyme system is very similar to the mixed function oxidase system present in microsomes. The compounds studied as substrates for this system were the volatile anesthetics, halothane (1,1,1-trifluoro-2-bromo-2-chloroethane) and methoxyflurane (2,2-dichloro-1,1-difluoroethyl methyl ether). Since each of these materials contains several halogens, it was essential to study in a more exact manner the dehalogenation of a given halogen and the conditions required for the optimum dehalogenation. By this study and others to follow, we hope to gain insight into the mechanisms of dehalogenation.

The present communication reports a study of the dechlorination of a series of chlorinated ethanes and propanes. These were selected in an attempt to study the effect of the number of chlorines per carbon and adjacent carbon substitution on the ability of the enzyme system to dechlorinate.

MATERIALS AND METHODS

Materials

The synthesis of the ^{36}Cl-chloroethanes and propanes was carried out in the Halogens Research Laboratory of the Dow Chemical Company. The procedures used for this are the subject of another publication.

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† Present address: Mayo Clinic, Rochester, Minn. 55901.

‡ Present address: Michael Reese Hospital, Chicago, Ill.

The separation and purification of the chlorinated hydrocarbons were achieved by gas chromatography. The final product was better than 99·9 per cent pure in all cases.

Methods

Assay of dechlorination in vitro. The *in vitro* assay of the enzymatic dechlorination was run as previously described.[2] Male rats weighing 120–200 g were used exclusively in this study. The complete incubation system contained: 2 ml microsomes in tris buffer, pH 7·4; NADP, 0·3 mM; glucose 6-phosphate, 1·5 mM; glucose 6-phosphate dehydrogenase, 1 unit; and 1 ml of cell supernatant fraction. These were added to the main compartment of a Warburg flask and then the flask was flushed with oxygen prior to sealing. The substrate was added either to the side arm dissolved in propylene glycol or directly to the incubation medium. The latter technique was used for the collection of the data in this report. Control incubations were run omitting NADP. Incubations were carried out for periods of varying lengths up to 1 hr, at which time aliquots (0·10 ml) were taken for determination of the total radioactivity present in the medium. This was necessary, since not all the substrate added either dissolved in or remained in the medium; but a partitioning occurred between the undissolved material, the medium, and the atmosphere above the medium. The incubation was then stopped with the addition of nitric acid, and the inorganic chloride was assayed and counted as previously described.[3]

Tissue preparation. The various tissue preparations used in this study were prepared from rat, rabbit and guinea pig liver. A 25% liver homogenate was prepared either in 0·25 M sucrose or in tris buffer, 0·05 M, pH 7·4 or in KCl, 1·15%. These homogenates were centrifuged at 9000 *g* (tris and KCl) or 12,500 *g* (sucrose) and the precipitate was discarded. The supernatant of this centrifugation was then centrifuged at 105,000 *g*. The pellet was resuspended in tris buffer at the indicated pH and the supernatant was saved.

pH Studies. The tissue was prepared as above, except that the tissue homogenate was prepared in buffers at various pH values. Tris buffer, 50 mM, was used to cover the pH range from pH 7·4 to 9. Protein concentrations were determined using the method of Lowry *et al.*[4]

Enzyme induction studies. Enzyme induction studies were performed by pretreating the animals for 3 days with sodium phenobarbital, 40 mg/kg, methylcholanthrene, 20 mg/kg, benzpyrene, 20 mg/kg, or by exposing the animals to vapors of methoxyflurane or trichloroethane. In the latter technique, the animals were exposed for 5 days, 7 hr per day, to 500 ppm of the vapors of either methoxyflurane, 1,1,1-trichloroethane or 1,1,2-trichloroethane. The animals were killed 24 hr after the last exposure or treatment and the livers were removed and used as indicated above.

RESULTS

It has previously been determined* that in the case of 1,1,2-trichloroethane the products of this dechlorination were mono- and dichloroacetic acid and mono- and dichloroethanol, and of these the monochloroethanol was the major metabolite. However, because of the multiplicity of metabolites, it was necessary to follow this dechlorination by measuring the release of inorganic chloride, since this proved to be a more precise method.

* R. A. Van Dyke and C. G. Wineman, unpublished results.

The results of the study of the dechlorination of the chlorinated ethanes is shown in Table 1. It is noteworthy that the dechlorination of these compounds is enhanced if the carbon atom containing the chloride had one hydrogen. This fact is also evident from the data in Table 2, which lists the chloropropanes studied and the percentage of chlorine-36 dechlorinated enzymatically. The percentage of dechlorination is based on the ratio of the radioactivity present in organic chloride to that present in the incubation medium at the conclusion of the experiment.

TABLE 1. DECHLORINATION *in vitro* OF CHLORINATED HYDROCARBONS BY RAT LIVER MICROSOMES*

Chlorinated material added to incubation	$\%^{36}Cl$ enzymatically removed
CH_3CH_2Cl	<0·5§
CH_3CHCl_2	13·5
CH_3CCl_3	<0·5
CH_2ClCH_2Cl	<0·5
$CH_2ClCHCl_2$	9·8
CH_2ClCCl_3	0·8
$CH_2Cl_2CHCl_2$	6·0
$CHCl_2CCl_3$	1·7
CCl_3CCl_3	3·9§
CHClCHCl	0·7
CCl_2CCl_2	<0·5

* Incubation medium consisted of: microsomal suspension, 2 ml; 5 mg protein/ml in tris buffer, pH 7·4; 1 ml of 105,000 *g* supernatant; NADP, 0·3 mM; glucose 6-phosphate, 1·5 mM; glucose 6-phosphate dehydrogenase, 1 unit. Final volume, 3·1 ml; 1 μl of the chlorinated hydrocarbon was added to the incubation medium. Incubations were carried out for 30 min in sealed Warburg vessels. Control incubations contained complete system with the chlorinated hydrocarbon, omitting the NADP. The incubations were stopped with nitric acid and the inorganic chloride was isolated and counted. The percentage of ^{36}Cl removed is based on the chloride-36 isolated as a percentage of the total radioactivity remaining in the incubation medium at the conclusion of the experiment.

† Uniformly labeled with chlorine-36.

‡ Results are averages of duplicate assays from at least six rats.

§ High control values; see text.

There was a considerable amount of dechlorination of the hexachloroethane and the ethyl chloride in the absence of NADP. This may reflect an alternate pathway of metabolism or a nonenzymatic breakdown. All the other compounds tested showed no evidence of dechlorination in the absence of NADP. In view of the fact that 1,1,2-trichloroethane proved to be one of the best substrates for this enzyme system, the majority of the following studies were carried out with it.

A major problem in this study was the fact that the compounds used as substrate varied in volatility from a boiling point of 12° to a sublimation point of 187°. Thus

TABLE 2. METABOLISM *in vitro* OF CHLOROPROPANES BY RAT LIVER MICROSOMES*

Chloropropane added to incubation	% Enzymatically dechlorinated	Chloropropane added to incubation	% Enzymatically dechlorinated
1-Chloropropane	3·2	1,1-Dichloropropane	24·6
1,2-Dichloropropane	5·8	1,1,2-Trichloropropane	40·8
2-Chloropropane	5·2	2,2-Dichloropropane	2·5

* See Table 1 for description of incubation.

the rate of diffusion either out of the side arm of the Warburg flask and into the medium or throughout the medium if added directly, would vary tremendously. It was determined that partitioning occurred much more rapidly if the material were added directly to the incubation medium and, thus, higher rates of metabolism were achieved much quicker. Table 3 shows the rate of release of inorganic chloride from ^{36}Cl-1,1,2,2-tetrachloroethane and ^{36}Cl-1,1,2-trichloroethane with time when added directly to the medium. It is apparent that the rates increase in the first 10 min, which is probably a reflection of the rate of diffusion throughout the medium.

TABLE 3. INITIAL RATE OF DECHLORINATION REACTION*

Time of incubation (min)	Substrate	
	1,1,2-Trichloroethane	1,1,2,2-Tetrachloroethane
3	2·9	1·8
5	5·5	3·0
10	8·5	4·5
15	8·0	4·3

* Data are presented as counts/min/mg of protein of chloride-36 released. Average of duplicate runs of three experiments. Substrate (2 μl) was added to each flask containing microsomes, cell supernatant and an NADPH generating system (see Table 1).

A comparison of the enzymatic dechlorination of the two isomers of trichloroethane is shown in Table 4. It is evident from these data that the 1,1,1-trichloroethane is dechlorinated in trace amounts while the 1,1,2-trichloroethane acts as a good substrate for the dechlorinating system. Table 4 also contains data pertaining to the induction of the dechlorinating system. The exposure of the rats to methoxyflurane vapors causes an induction of the dechlorinating system of 1,1,2-trichloroethane, but not of 1,1,1-trichloroethane. On the other hand, exposure to the 1,1,1-trichloroethane vapor had no effect on the dechlorinating system. The exposure to 1,1,2-trichloroethane was not successful because of the very high toxicity of this material, which resulted in the death of all the animals before induction could be demonstrated.

TABLE 4. EFFECT OF ANIMAL PRETREATMENT ON THE ENZYMATIC DECHLORINATION *in vitro* OF THE TRICHLOROETHANE ISOMERS*

Animal pretreatment	Inorganic chloride-36 released/mg protein	
	1,1,1-Trichloroethane added to incubation	1,1,2-Trichloroethane added to incubation
None	3·1 ± 0·3	65 ± 0·9
1,1,1-Trichloroethane exposure	4·0 ± 0·7	67 ± 4·8
Methoxyflurane exposure	3·5 ± 0·7	86 ± 3·8

* Incubations were carried out according to the method in Table 1, with the exception that these were 1-hr incubations. Each number represents the average of duplicate incubations from eight animals ± S.E. The pretreatment with 1,1,1-trichloroethane and methoxyflurane was accomplished by exposing rats for 7 hr daily for 3 days to 500 ppm of the vapor.

A comparison of the effects of pretreatment of the animals with phenobarbital, methylcholanthrene and benzpyrene is listed in Table 5. Methylcholanthrene does not induce this activity, while benzpyrene and phenobarbital do.

Since it is necessary to have present in the incubation medium some 105,000 *g* supernatant, studies were performed to determine if this activity was inducible. Table 6 lists the results of this study. It is evident that this supernatant factor is not inducible.

TABLE 5. INDUCTION OF HEPATIC DECHLORINATION SYSTEM*

Inducing agent	Per cent of control
Control	100
Phenobarbital	305
Benzpyrene	166
Methylcholanthrene	95

* Incubations were carried out using the method in Table 1. One μl 1,1,2-trichloroethane was added directly to the incubation medium. Incubations were carried out for 30 min. Control animals received either saline or corn oil, i.p. Phenobarbital was given at 40 mg/kg for 3 days. Benzpyrene and methylcholanthrene were administered at 20 mg/kg for 3 days. The livers were removed 24 hr after the last injection and perfused with cold saline.

The problem of the nature of the supernatant activity still exists. In Table 7 are listed the various results obtained in attempting to find out more about this factor. The data presented in Tables 1 and 2 utilized 1 ml of 105,000 *g* supernatant; in Table 7, supportive data indicate that 1 ml is in excess of that needed for optimum activity. It should also be noted that the addition of other proteins does not replace the supernatant activity, as is the case with other drug metabolisms.[5] One other interesting point

indicated in Table 7 is that by washing the microsomes the activity of the microsomes alone is lowered. This may reflect the ease with which this factor is either destroyed or washed from the microsomes.

The pH optimum was determined and the results are presented in Table 8. The peak activity occurred at pH 8·2, although the pH optimum was fairly broad, covering the range of pH 8·0–8·4.

TABLE 6. EFFECT OF ENZYME INDUCTION ON THE CELL SUPERNATANT REQUIREMENT FOR THE ENZYMATIC DECHLORINATION OF $_{22}$Cl-1,1,2-TRICHLOROETHANE*

Protein source†	Inorganic chloride-36 released (counts/min/mg protein)
M-C	4·9
M-C + S-C	27·5
M-T	10·0
M-T + S-T	61·2
M-C + S-T	32·5
M-T + S-C	70·6

* See Table 1 for details of incubation.

† M-T = microsomes from livers induced with phenobarbital;
S-T = 105,000 *g* supernatant from livers induced with phenobarbital;
M-C = microsomes from control animals;
S-C = 105,000 *g* supernatant from control animals.

TABLE 7. SUMMARY OF FACTORS AFFECTING THE DECHLORINATION OF 1,1,2-TRICHLOROETHANE*

System	Relative activity
Microsomes + 1 ml supernatant	100
Microsomes	61
Microsomes + 0·5 ml supernatant	100
Microsomes + 0·2 ml supernatant	70
Microsomes + bovine serum albumin	44
Microsomes + 1 ml "aged" supernatant (stored 6 hr at 4°)	81
Microsomes + 1 ml supernatant (NADH substituted for NADPH)	46
Microsomes washed with tris buffer (no supernatant added)	22
Microsomes + 1 ml heated (80°) supernatant	19
Microsomes + glutathione (10^{-4}M)	73
Microsomes + 1 ml supernatant + glutathione (10^{-4}M)	99

* Data are expressed as relative activity to microsomes + 1 ml supernatant. All incubations were carried out for 30 min. All flasks contained NADPH generating system (see Table 1). NADP was omitted from control flasks.

This enzyme activity is not highly sensitive to inhibition by carbon monoxide, as indicated in Table 9. This is not as highly sensitive to inhibition by CO as has been found for other drug-metabolizing systems.[6]

TABLE 8. EFFECT OF pH ON ENZYMATIC DECHLORINATION *in vitro**

pH	Chloride-36 released (counts/min/mg protein)
7·6	96·5
8·0	103·8
8·4	130·8
8·8	68·4
9·1	57·6

* Substrate used was ^{36}Cl-1,1,2-trichloroethane. See Table 1 for conditions of incubation, with the exception that these were 1-hr incubations.

TABLE 9. EFFECT OF CO ON THE ENZYMATIC DECHLORINATION *in vitro* OF 1,1,2-TRICHLOROETHANE*

Percentage composition of gas used to flush incubation flasks			Inorganic chloride-36 released (counts/min/mg protein)
O_2	N_2	CO	
25	75		149
25	50	25	144
25	37·5	37·5	139
25	25	50	123
25		75	117
100			147

* See Table 1 for conditions of incubation.

DISCUSSION

There is a growing list of halogenated compounds which can be dehalogenated by enzymes from various sources. Goldman *et al.*[7,8] have reported the isolation and partial purification of three bacterial halidohydrolases. These enzymes catalyze the dehalogenation of halogenated fatty acids. Several dehalogenations have been found to occur as the result of the mammalian mixed function oxidase systems.[9–12] In these cases, it has been found that fluoride is released from aromatic compounds, while chlorine, if present, undergoes a migration in certain cases rather than a release.

The data contained in the present report indicate that the chloroethanes and propanes undergo a dechlorination by an enzyme system which is similar in several respects to the hepatic microsomal mixed function oxidase system used for the metabolism of a variety of drugs.[13] As indicated in the present study, the dechlorinating system is inducible by phenobarbital or benzpyrene, requires oxygen and NADPH. In

contrast to most of the other mixed function oxidase systems, however, the dechlorinating system requires a factor from the soluble portion of the liver homogenate for optimal dechlorinating activity. In addition, the dechlorinating system is apparently either only slightly dependent on cytochrome P-450 or the P-450 is not the rate-limiting step in the overall dechlorination reaction. Neither of these facts is unique to microsomal oxidases, since reports have appeared that a pteridine cofactor found in the soluble portion of the cell is required for the optimum hydroxylation of phenylalanine[9] and the oxidation of glycerol ethers[14] by a microsomal mixed function oxidase system. Also, Sladek and Mannering[15] have reported that it is possible that the ethylmorphine *N*-demethylation reaction either does not utilize P-450 or that it is not the rate-limiting step in the overall reaction.

Several reports have appeared in recent years describing an enzyme called alkyl-S-transferase,[16] which is soluble and catalyzes the substitution of a halogen on a halogenated aliphatic by glutathione through the sulfhydryl group. This results in the release of the halide and the eventual formation of mercapturic acid. The system for dechlorinating reported in the present communication is considerably different in that the dechlorinating enzymes are entirely microsomal and the presence of gluthathione does not stimulate the enzyme activity and therefore is not the supernatant factor in this case.

The mechanism of oxidative enzymatic dechlorination of chlorinated aliphatic hydrocarbons is under investigation.

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