Dechlorination of halocarbons by microsomes and vesicular reconstituted cytochrome P-450 systems under reductive conditions

A G SALMON,¹ J A NASH,² C M WALKLIN,³ AND R B FREEDMAN³

From the TUC Centenary Institute of Occupational Health,¹ London School of Hygiene and Tropical Medicine, London WC1E 3HT, Central Toxicology Laboratory,² Imperial Chemical Industries plc, Macclesfield, Cheshire, and Biological Laboratory,³ University of Kent, Canterbury, UK

ABSTRACT A spectrophotometric assay of the reductive dechlorination of halocarbons was developed and used to determine the kinetic characteristics of dechlorination of a range of haloethanes catalysed by microsomes from rat and rabbit liver. Analysis of the typical reaction of hexachloroethane shows that the reaction is catalysed by cytochrome P-450 and results in the formation of olefinic products as well as less chlorinated alkanes: in other respects the reaction resembles that known to occur with carbon tetrachloride. The dechlorination of haloethanes catalysed by a vesicular reconstituted system of cytochrome P-450 enzymes from rabbit liver was also studied and found to be similar to that catalysed by microsomes: both reductase and a phenobarbital inducible form of cytochrome P-450 were essential. There is no substantial dependence of maximum dechlorination rate on compound structure, suggesting that the reduction of substrate is not the rate limiting step in the overall reaction. The main factor in determining the apparent binding constant to the enzyme is the partition coefficient into the lipid membrane, as assessed by calculated log P values.

The metabolism of halogenated hydrocarbons commonly involves dehalogenation, which in many cases is dependent on microsomal cytochrome P-450. Van Dyke and Wineman showed that the dechlorination of chloroalkanes was dependent on NADPH and inhibited by CO; activity was also inducible by phenobarbital but not by 3-methylcholanthrene.¹ Analysis of the reactivity of halocarbons as a function of their structure has been attempted on the assumption of a single mechanism.² We have shown previously, however, the necessity of considering more than one mechanism in order to explain the behaviour of various compounds that have been studied *in vitro*.^{3,4} This is in agreement with the observations of products and mechanisms for those halocarbons that have been studied in detail: these data have been reviewed recently.⁵ It seems that most dechlorination reactions are oxidative in character, involving an initial attack by an oxygenated complex of cytochrome

P-450 on a hydrogen atom, resulting in rearrangement of the product with the loss of a halogen. Nevertheless, at least one other important mechanism of dechlorination exists, which is observed when chemical structure or reaction conditions prevent the oxidative route. This mechanism, of which the metabolism of carbon tetrachloride is the best known example, involves reductive generation of a radical and direct release of a halide ion.

The objectives of this study were: firstly, to define a simple and reliable assay for the reductive reaction, based on the provision of a suitable anaerobic reaction medium.⁶ This needed to be sufficiently reproducible to allow proper kinetic analysis of the results. Secondly, to apply the assay and kinetic analysis to a range of haloethanes to investigate the structure/reactivity relationship for the reductive reaction. Thirdly, to test the role of cytochrome P-450 isoenzymes and NADPH:cyt P-450 reductase directly by studies using reconstituted systems. Previous studies have reported reconstitution of reductive dechlorination of carbon tetrachloride and haloethane in non-vesicular systems comprising

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cytochrome P-450, reductase, lipid, and detergent.^{7,8}

To allow the use of the better defined and more easily prepared rabbit P-450 enzymes, the reaction was studied using both rabbit liver microsomes, with and without induction by phenobarbital and β -naphthoflavone, and also the rat liver microsomes that have been the subject of most previous work. These data were then compared with those obtained with purified cytochrome P-450 enzymes isolated from rabbit liver in a reconstituted system of vesicles containing reductase, lipid, and cytochrome. We have investigated the progress of this reaction under favourable conditions in vitro for a series of compounds that were expected to be susceptible to this process. Observed reactivities were correlated with structural features of the reacting species. We have also examined the metabolites formed in vitro from a typical compound, hexachloroethane. Further investigations performed with this compound were designed to examine the effects of the mixed function oxidase inhibitors carbon monoxide and SKF 525A on the reaction.

Methods

PREPARATION OF MICROSOMES

Rat microsomes were prepared from the livers of normal (non-induced) male rats (Alderley Park strain, Wistar derived). The method of preparation has been described.⁹ Food was withdrawn 24 hours before death, and the washed microsomes were stored under argon at -70°C . Rabbit microsomes were prepared by the method of van der Hoeven and Coon¹⁰ from the livers of 3 month old male New Zealand White rabbits and stored at -70°C . For non-induced microsomes, the animals had access ad libitum to plain tap water for drinking, but for phenobarbital induction this was replaced by tap water to which sodium phenobarbital (0.1% w/v) was added. Induction by β -naphthoflavone was achieved by intraperitoneal injections daily for three days at 20 mg/kg as a 1% (w/v) suspension in corn oil.

All chemicals were of AnalaR grade or next highest available purity, and were obtained from BDH, Poole, Dorset, unless noted to the contrary. (Tris buffer used was "Trizma" base enzyme grade material from Sigma (London) Chemical Co, Poole.) Ethoxyresorufin and resorufin were gifts from Dr T C Orton, ICI Pharmaceuticals Division. Benzphetamine HCl was a gift from Dr G G Gibson, University of Surrey. β -naphthoflavone was purchased from Aldrich Chemical Co, Gillingham.

SPECTROPHOTOMETRIC ASSAY OF DEHALOGENATION

Stored microsomes were thawed gently and diluted with buffer (Tris 50 mM, EDTA 1mM, pH 7.5) which had been deoxygenated by bubbling with nitrogen for one hour. The reaction mixture was prepared with the following components, also in deoxygenated buffer: microsomes (1 mg/ml), D-glucose 30 mM, Catalase, 2000 units/ml (from bovine liver, purchased from Sigma), glucose oxidase 5 units/ml (from *Aspergillus niger*, type V, also purchased from Sigma), NADPH 500 μmol . Samples of the resulting preparation were placed in sealed cuvettes under nitrogen. All transfers were carried out under nitrogen with flushed syringes, containers being sealed with silicone rubber septa or stoppers. The cuvettes were placed in a Beckman Acta MVI or a Perkin Elmer 356 spectrophotometer fitted in both cases with a scattering sample attachment. Absorbance at 340 nm was monitored, a "baseline" rate of change without substrate being established first. The substrate was added to the sample cuvette as a solution in 1 μl spectroscopic grade ethanol. The change in absorbance at 340 nm was observed as a function of time, and the initial rate determined. Spectrophotometric assays were performed in a similar manner with a reconstituted cytochrome P-450 system (see below). The kinetic parameters K_m and V_{max} were determined by a computer program¹¹ which uses the direct linear plot¹² and also fits regression lines to the three standard linear transformations of the Michaelis-Menten Equation. The halocarbon substrates were obtained from Aldrich Chemical Co, Gillingham.

To study the effect of SKF525A on the reaction of hexachloroethane, the compound (a gift from Smith, Kline and French, Welwyn) was added in buffer. In the similar experiment with carbon monoxide, the reaction mixture was saturated with the gas by bubbling for two minutes.

IDENTIFICATION OF REACTION PRODUCTS

For aerobic incubations, 25 ml flasks sealed with silicone septa were prepared, containing the following reagents in a total volume of 10 ml: KCl (75 mmol), D-glucose-6-phosphate (5 mmol), NADP⁺ (250 μmol), nicotinamide (4.5 mmol), glucose-6-phosphate dehydrogenase (0.25 units/ml), in Tris buffer (as above). For anaerobic incubations, an oxygen scavenging system was incorporated in the reaction system. This consisted of D-glucose, catalase, and glucose oxidase, as used in the spectrophotometric assay. All solutions used in anaerobic incubations were deoxygenated before use, and the headspace of the flask was purged with nitrogen.

^{14}C -hexachloroethane (final concentration $2\ \mu\text{M}$, $41.44\ \mu\text{Ci}$ radioactivity) was added in $20\ \mu\text{l}$ ethanol to the liquid phase and the flasks incubated at 37°C in a shaking water bath. Flasks were sampled immediately after injection of substrate and after 145 minutes. Control incubations with boiled microsomes were run concurrently. The headspace was sampled ($1\ \text{ml}$) with a gas-tight syringe, and analysed by gas liquid chromatography. Two columns were used as necessary to separate and identify the labelled metabolite peaks. These were a $1.5\ \text{m} \times 2\ \text{mm}$ id glass column packed with 5.5% OV101 on Supelcoport (100–200 mesh), maintained at 80°C , and a $2\ \text{m} \times 2\ \text{mm}$ id glass column packed with 60/80 Carbowax B/1% SP1000 (Supelco Inc), maintained at 175°C . Gas chromatography materials were obtained from Phase Separations Ltd (Clwyd). A Pye 104 gas chromatograph with flame ionisation detector and a radiochemical detector (ESI Nuclear) was used. Metabolite peaks were identified by comparison of retention times and by cochromatography with authentic standards. The gas-tight syringe was purged with nitrogen and checked for carry over between samples.

The radiolabelled hexachloroethane ($21.5\ \text{mCi/mmole}$) used was obtained from Physics and Radioisotopes Services, ICI Petrochemicals and Plastics Division, Billingham. This material was purified by gas liquid chromatography and had a radiochemical purity of better than 98%. No impurity peaks were detected on analysis of the material by gas liquid chromatography, either with the flame ionisation or radioactivity detectors.

RECONSTITUTED SYSTEMS

Purified preparations of rabbit cytochromes P-450 (LM2 and LM4) were derived by a procedure involving polyethylene glycol fractionation of cholate solubilised, pyrophosphate washed microsomes, followed by ion-exchange chromatography on DEAE-Sephacel, adsorption chromatography on spheroidal hydroxyapatite and calcium phosphate (all in the presence of Renex 690), treatment with Amberlite XAD-2 to remove detergent, and chromatography on Octyl-Sepharose 4B. The method is modified from the procedures of van der Hoeven and Coon¹⁰ and Wolf *et al.*⁷: full details are given by Walklin.¹³ NADPH:cytochrome P-450 reductase was purified by the method of French and Coon.¹⁴ Reconstruction of these proteins in vesicles with dilaurylglycerolphosphatidylcholine (Fluka AG, Buchs, Switzerland, synthetic, 98% pure) was by the cholate gel filtration method.¹⁵ The vesicles eluted at the void volume from columns of Sephadex G50 (Medium) and electron microscopy showed them to be roughly spherical, having diameters in

the range 150–200 nm. SDS-polyacrylamide gel electrophoresis analysis showed that close to 100% of the added enzymes had been incorporated into the vesicles.

OTHER ASSAYS

Mixed function oxidase activities towards standard substrates were assayed: ethoxyresorufin O-deethylation,¹⁶ and benzphetamine N-demethylation.¹⁷ Protein concentrations were measured by the method of Lowry *et al.*¹⁸ modified for use with membrane proteins,¹⁹ using crystalline bovine serum albumen as a standard. Cytochrome P-450 content was assayed spectrophotometrically²⁰: the specific cytochrome P-450 contents of the purified preparations used in this work were: LM2 $12.5\ \text{nmol/mg}$, LM4 $11.7\ \text{nmol/mg}$. NADPH:cytochrome P-450 reductase was assayed using cytochrome c as oxidant²¹; the stock preparation used here had an activity of $52.5\ \text{units/mg}$. (One unit catalyses the reduction of $1.0\ \mu\text{mol}$ cytochrome c per minute: $0.3\ \text{M}$ phosphate buffer pH 7.7, 30°C .)

Results and discussion

The anaerobic assay system allowed the reliable determination of rates of cytochrome P-450 dependent reductive dechlorination reactions catalysed by liver microsomes. In the absence of added substrate the rate of NADPH oxidation was negligible, and on addition of substrate it proceeded linearly for at least two minutes. Using rat liver microsomes with hexachloroethane as substrate, 98% was inhibited by saturation of the assay mixture with CO and 37% inhibited by $100\ \mu\text{mol}$ SKF 525A. When phenobarbital induced rabbit liver microsomes were used the CO inhibition approached 100%.

The incubation of microsomes ($1.377\ \text{mg/ml}$, $1.4\ \text{nmoles P450 per mg protein}$) and an NADPH generating system with hexachloroethane followed by gas chromatographic analysis of the headspace showed the formation of tetrachloroethane as the major product, under conditions of normal oxygen partial pressure and also anaerobically (fig 1). The rate under anaerobic conditions seemed to be greater than in the presence of oxygen: although exact quantitation of total product is not possible using the headspace method, the amount of product formed under aerobic conditions appeared to be between 10% and 20% of that formed under anaerobic conditions. A second product, trichloroethylene, was observed under aerobic conditions in small quantities, but was produced in larger amounts in the anaerobic incubation. The other product observed was pentachloroethane. Under aerobic conditions the peak corresponding to this compound was no

Fig 1 Gas liquid chromatography of headspace products of a dechlorination reaction catalysed by rat liver microsomes under anaerobic conditions. Conditions of incubation were as described in text, and chromatography was on a column consisting of 5% OV101 on Supelcoport.

larger than that seen in the boiled microsome control incubation, but a significant increase in the amount of this material in the headspace (150%) was seen under anaerobic conditions. The presence of significant amounts of material corresponding to products in the boiled microsome control incubations was noted, but this observation was not repeated in control incubations lacking only substrate or microsomes. It was therefore concluded that in addition to the enzyme catalysed reaction, a small catalytic effect is shown by a heat-stable or heat generated component of the boiled microsomes, possibly Fe^{+++} . Substantial amounts of radioactivity were shown to remain in the aqueous phase after the complete incubations, some but not all of which was

Fig 2 Reductive dechlorination of pentachloroethane catalysed by rat liver microsomes. Initial velocities observed in spectrophotometric assay described above are displayed as a function of substrate concentration.

unchanged hexachloroethane ^{-14}C .

The inhibitor effects and the nature of the products indicate that the reaction being assayed spectrophotometrically is a cytochrome P-450 dependent reductive dechlorination. It has been concluded on the basis of similar observations²² that these products resulted from a two electron reduction of the substrate. The reaction resembles the reductive dechlorination of carbon tetrachloride except that the intermediate radical product is readily suscept-

Table 1 Kinetic parameters for reductive dechlorination of halocarbons by control rat liver microsomes

Compound	K_m (μM)	$V_{\max}$ nmol NADPH/min (mg/protein)	$V_{\max}$ nmol NADPH/min nmole P-450
Carbon tetrachloride	9.6	3.35	2.15
Hexachloroethane	6.0	3.55*	2.41
Pentachloroethane	25.1*	4.35*	3.35
1,1,1,2-tetrachloroethane	20.4*	3.41	2.62
1,1,1,2-tetrachloroethane	(a)	(a)	(a)
1,1,1-trichloroethane	(b)	(b)	(b)
1,1-dichloroethane	(a)	(a)	(a)
1,2-dichloroethane	(a)	(a)	(a)
1,1,1-trichloro-2,2,2-trifluoroethane	11.7	1.78	1.37
1,1,1,2-tetrachloro-2,2-difluoroethane	25.9	4.49	3.45

Kinetic parameters were derived from Direct Linear, Eadie-Hofstee, and Lineweaver-Burk plots of the data. The Lineweaver-Burk plot is known to be most prone to error when there is experimental scatter in data. Parameters quoted are those obtained from a regression line of best fit to data plotted as an Eadie-Hofstee plot; except in a few cases (*) the values obtained from Direct Linear plot are within 10% of values quoted.

(a) Rates of NADPH oxidation observed were too low to allow determination of kinetic parameters.

(b) NADPH oxidation was observed at a low rate, but protein precipitation interfered with measurements at substrate levels high enough to allow determination of kinetic parameters.

Table 2 Water: octanol partition data

Compound	Log P
Carbon tetrachloride	2.96
Hexachloroethane	4.62
Pentachloroethane	3.69
1,1,1,2-tetrachloroethane	3.05
1,1,2,2-tetrachloroethane	2.72
1,1,1-trichloroethane	2.50
1,1-dichloroethane	1.80
1,2-dichloroethane	1.48
1,1,1-trichloro-2,2,2-trifluoroethane	3.26

Log P is a calculated octanol:water partition coefficient, derived by Mr P J Taylor, ICI Pharmaceuticals Division, using the method of Leo *et al.*²³

ible to further reduction to the olefin in the case of the two-carbon substrates, whereas for carbon tetrachloride this material is not so readily subject to further metabolism and tends to accumulate. For both the haloethanes and carbon tetrachloride this reductive reaction, although more readily detected under anaerobic conditions, continues at a substantial rate even at the atmospheric partial pressure of oxygen, which is higher than that to be expected in some tissues—for example, those parts of the liver acinus closer to the central vein. It is therefore supposed that this type of reaction is of physiological and pathological importance.

The rate of reductive dechlorination by control rat liver microsomes was assayed spectrophotometrically with a large number of haloethane substrates, at concentrations between 2 and 500 μmol . In general the data are in good agreement with the Michaelis-Menten equation (see fig 2). These data were analysed to provide values of K_m and $V_{\max}$,

Fig 3 Reductive dechlorination of 1,1,1-trichloroethane catalysed by purified rabbit liver cytochrome P-450 (LM2). Conditions for the spectrophotometric assay were as described for vesicular reconstituted systems, initial velocities being presented as a function of substrate concentration.

Table 3 Kinetic parameters for reductive dechlorination of chloroethanes by a vesicular reconstituted system

Compound	K_m (μM)	$V_{\max}$ nmol NADPH/min nmole P450
Hexachloroethane	50	2.39
Pentachloroethane	104*	1.48
1,1,2,2-tetrachloroethane	119	1.66
1,1,1-trichloroethane	360	4.33
1,1-dichloroethane	802	1.27

See legend to table 1.

which are reported for a number of substrates in table 1. Similar observations were made for rabbit liver microsomes, although a rather higher absolute rate was observed, especially after phenobarbital induction.

With both rat and rabbit microsomes the major finding is that there is limited variation in $V_{\max}$, but considerably greater variation in K_m . The lack of dependence of $V_{\max}$ on structural features of the haloethanes suggests that in this reductive dechlorination the substrate reaction is not rate limiting. The interpretation of K_m values for the interaction of non-polar substrates with membrane bound enzymes is complicated by the partition of the substrate between the aqueous and the membrane phases; the values obtained neglect this partition and are therefore not true measures of the dissociation constant describing the equilibrium between free substrate and enzyme/substrate complex. Hence it is dangerous to attempt structure/activity interpretations of these crude K_m values. In general it is found that the K_m values decrease with increasing extent of chlorination of the substrates; this may reflect the increasingly non-polar nature of the more highly substituted substrates, and hence their greater partition into the membrane phase and their concentration in the environment of the enzyme. The polarities of the various compounds are illustrated in terms of their water-octanol partition coefficients (measured or derived) given in table 2. If it is assumed that substrate partition between bulk aqueous phase and membrane as determined by these constants and that substrate in the membrane phase then equilibrates with the substrate binding site on the cytochrome, the "true" K_m describing this binding equilibrium is given by:

$$K_m = \frac{K_m^{\text{app}} \cdot P \lambda}{1 + P \lambda}$$

where K_m^{app} is the value derived directly from kinetic studies, P is the partition coefficient and λ is the volume fraction occupied by the membrane phase.^{24 25} Using this approach the true K_m values

for the chloroethanes with rat liver microsomes are in the region of 16 mmol.

Involvement of particular cytochrome P-450 species in the reductive dechlorination is suggested by studies with liver microsomes from animals pretreated with inducers. Microsomes from β -naphthoflavone induced rabbits did not catalyse the reaction at a measurable rate with any of the six haloethanes tested, and microsomes from control rabbits gave a measurable rate of reaction at high concentrations of hexachloroethane only. On the other hand, those from phenobarbital induced rabbits catalysed the reaction with several compounds. K_m values for these microsomes ranged from $<5 \mu\text{mol}$ for hexachloroethane to about $80 \mu\text{mol}$ for 1,1,2,2-tetrachloroethane.

These findings suggest that the reductive dechlorination reaction is preferentially catalysed by a cytochrome P-450 isoenzyme that is induced by phenobarbital, possibly the major phenobarbital inducible form. This hypothesis was tested by the isolation from rabbit liver microsomes of the major phenobarbital induced isoenzyme (LM2), and the isolation of the major aromatic hydrocarbon inducible form (LM4) for comparison. These proteins were isolated rapidly and in good yield by a new combination of chromatographic techniques described fully elsewhere.¹² The purified cytochrome P-450s were reconstituted with and without rabbit liver NADPH:cytochrome P-450 reductase, in defined lipid bilayer vesicles of dilauryl phosphatidyl choline. Vesicles containing LM2 and reductase were active in the NADPH dependent demethylation of benzphetamine, but not the NADPH dependent de-ethylation of ethoxyresorufin, whereas the opposite was true of vesicles containing LM4 and reductase. No mixed function oxidase activities were observed with vesicles containing cytochrome P-450 alone or reductase alone.

Vesicles containing cytochrome P-450 LM2 and reductase were active in the NADPH dependent reductive dechlorination of haloethanes. Figure 3 shows kinetic data obtained in the reaction with 1,1,1-trichloroethane: kinetic data for a range of substrates are shown in table 3. Interestingly, vesicles containing LM2 and reductase catalyse reactions of a slightly wider range of haloethane substrates at measurable rates than do phenobarbital induced microsomes (table 3 cf table 1). Comparison of the kinetic data with various substrates shows that again there is little variation in $V_{\max}$ (3-fold), and considerably greater variation in the K_m (16-fold). As is the case with microsomes, K_m values decreased with increasing extent of halogen substitution. Adopting the approach described above to calculate "true" K_m values yields for all substrates

values in the region of 80 mM ($\pm 50\%$). Figure 4 shows the effect of $\log P$ in compensating for the variation in apparent K_m , assuming the substrates bind from the membrane phase. Thus in this system it appears that neither $V_{\max}$ nor the "true" K_m is strongly dependent on the nature of the substrate and that the rate determining step may be independent of substrate. No activity was observed with vesicles containing LM2 alone or reductase alone, nor with vesicles containing LM4 and reductase. The reaction is therefore catalysed only by certain cytochrome P-450 isoenzymes, and is entirely dependent on a flow of electrons from NADPH via NADPH:cytochrome P-450 reductase. The possibility that NADPH, cytochrome b_5 and NADPH:cytochrome b_5 reductase could also participate as electron donors to cytochrome P-450 was not investigated.

The data presented here are the first direct evidence that the reductive dechlorination pathway shown for a wide range of haloethanes is a specific property of certain cytochrome P-450 isoenzymes.

Fig 4 Variation of K_m with $\log P$ when calculated on basis of total substrate ($\bullet$), substrate present in the membrane phase ($\blacktriangle$), and substrate present in the aqueous phase ($\circ$), partitioning of substrate being calculated as described in discussion.

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