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THE REDUCTIVE METABOLISM OF HALOGENATED ALKANES BY LIVER MICROSOMAL CYTOCHROME P450*

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Abstract—Under anaerobic conditions various polyhalogenated alkanes (CCl_3–CCl_3, HCl_2C–CCl_3, CF_3–CCl_3, CCl_4, CF_3–$CHClBr$) stimulate the oxidation of NADPH by liver microsomal fractions. The participation of cytochrome P450 in the NADPH oxidation was shown by inducers and inhibitors of the monooxygenase system. The products of the reductive pathway of hexachloroethane were tetrachloroethene (99.5%) and pentachloroethane (0.5%). From pentachloroethane as substrate trichloroethene (96%) and tetrachloroethane (4%) were produced. The stoichiometry of NADPH oxidation and product formation was close to 1:1. There was a synergistic effect in the presence of NADPH and NADH for both hexa- and pentachloroethane. The influence of dioxygen and radical traps (RSH) on the formation of products from hexachloroethane with reduced cytochrome P450 has been investigated. The results indicate the possibility of a reductive *in vivo* metabolism of polyhalogenated alkanes even at physiological dioxygen concentrations. For the reductive dehalogenation of polyhalogenated alkanes by microsomal cytochrome P450 a reaction scheme is proposed: the reduction proceeds by two subsequent one electron reductions forming first a radical and then a carbanion. The carbanion can form an alkene via β-elimination of chloride.

Polyhalogenated aliphatic compounds are widely used as solvents and often have pronounced anaesthetic properties. They are generally toxic to the organism and their metabolic fate in the body is largely dependent on their chemical structure. It is now well established that certain biotransformations of polyhalogenated alkanes in liver result in toxic reactions which could be responsible for the liver damage observed [1]. Studies on the covalent binding caused by some halogenated alkanes clearly favour a reductive rather than an oxidative metabolism for the formation of reactive intermediates both *in vivo* and *in vitro* [2, 3].

There is direct evidence that polyhalogenated compounds can undergo reductive metabolism by microsomal cytochrome P450 *in vivo* and *in vitro* [3–5]. This resembles the reductive metabolism of nitroso-, azo-, nitro- and epoxy compounds [6–9].

Such a reductive function of cytochrome P450 is perhaps unexpected in view of the high affinity of the enzyme for dioxygen. However, it has been suggested that the dioxygen concentration in cells can be quite low [10], especially in the centre of the liver lobules, and this allows lipophilic reducible compounds with relatively high oxidation potentials to compete effectively with dioxygen at the active site of the various cytochrome P450 isoenzymes. The donation of one electron from cytochrome P450 to a polyhalogenated substrate, followed by a loss of halide ion, would give a carbon-centred radical as the first-formed intermediate. Indeed, the formation of the trichloromethyl radical during the metabolism of tetrachloromethane has been demonstrated by means of spin-trapping [11] and isotopic experiments [12]. Such radical species, in conjunction with dioxygen, could initiate lipid peroxidation and thereby cause membrane damages as the crucial event of hepatotoxicity.

Recent studies performed in our laboratories have suggested that cytochrome P450 can also donate a second electron to the trichloromethyl radical to give the carbanion. This may undergo α-elimination of a second halide to generate dichlorocarbene [12]. An alternative reaction pathway for a carbanion with more than one carbon atom would be β-elimination of a halide anion to form an alkene [13]. This reaction is described in the present paper taking hexa- and pentachloroethane as representative substrates. A preliminary report of some of this work has been made [14].

MATERIALS AND METHODS

Male Sprague–Dawley rats (100–150 g) treated with sodium phenobarbitone (PB) (80 mg/kg of body weight, intraperitoneal injection per day for 3 days) or 3-methylcholanthrene (3-MC) (20 mg/kg b. wt, injected intraperitoneally as a solution in olive oil daily for 2 days) were used.

Liver microsomal fractions were prepared according to Frommer *et al.* [15]. Protein was determined by the biuret method [16], and cytochrome P450 by the method of Omura and Sato [17]. Cytochrome b_5 was measured by the method of Strittmatter *et al.* [18].

All other chemicals used were obtained from regular commercial sources.

Incubations in 0.1 M Tris–HCl buffer (4 ml), pH 7.6 contained 1 mg/ml microsomal protein, 0.5 mM methanolic solution of the halogenated compound

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and an NADPH generating system (12 mM $MgCl_2$, 16 mM glucose-6-phosphate, 2 U glucose-6-phosphate dehydrogenase and 0.5 mM $NADP^+$). To obtain anaerobic conditions, dioxygen was removed from the system by repeated evacuation (3 times) and flushing with dinitrogen (5 min) before introduction of the substrate. After addition of $NADP^+$ the reaction was started by adding the substrate. The mixture was incubated at 30° for 10 min in a closed reaction vessel. The reaction was stopped by addition of 10% perchloric acid (0.4 ml) and extracted with 2 ml pentane. Analysis by g.l.c. was carried out on a 4% SE-30 glass column (2 m, 2 mm ID.) at 70° using a Hewlett Packard 5700 gas chromatograph equipped with an electron capture detector.

The rate of NADPH oxidation was recorded in stoppered 1 cm glass cuvettes containing 3 mg microsomal protein in 3 ml of 0.1 M Tris–HCl buffer, pH 7.6. After addition of NADPH (200 μM) and the substrate (0.5 mM) to the test cuvette the absorption change at 340 nm was recorded with time against a reference containing microsomes in an Aminco-DW 2 spectrophotometer in the split beam mode at 30°. An extinction coefficient of 6.22 $mM^{-1}\,cm^{-1}$ was used for calculating the rate of NADPH oxidation.

Assays with different concentrations of dioxygen were performed in 100 ml flasks closed by rubber stoppers. The reaction mixture contained 10 mg of microsomal protein in 10 ml 0.1 M Tris–HCl buffer pH 7.6 and an NADPH generating system (as above). The different ratios of dioxygen/dinitrogen were obtained by addition of distinct volumes of dioxygen to the N_2 atmosphere of the reaction vessel. The reaction was started by addition of the haloalkane (0.5 mM). After 5 min incubation at 25° and with vigorous shaking, 0.4 ml of 10% perchloric acid was added and the mixture extracted with 5 ml of n-pentane. Analysis by g.l.c. was carried out as described above.

RESULTS

Polyhalogenated alkanes can induce the oxidation of NADPH under anaerobic conditions in liver microsomal fractions. The rates of NADPH oxidation initiated by 9 polyhalogenated alkanes, are shown in Table 1. Of all compounds tested, hexa- and pentachloroethane gave the highest rates of NADPH oxidation in the range of 27–35 nmoles/mg protein/min in several microsomal preparations from phenobarbitone-pretreated rats. 1,2-Dichloro-, 1,1,2,2-tetrachloro-, 1,1,1-trichloroethane and trichloroethene failed to give a measurable rate of oxidation. In the presence of the known monooxygenase inhibitor carbon monoxide, 98 per cent inhibition of the substrate induced NADPH oxidation was established for the halogenated compounds investigated with the exception of 1,1,1-trifluorotrichloroethane and carbon tetrachloride which showed about 81 per cent inhibition.

Table 1. Rate of NADPH oxidation of halogenated alkanes with liver microsomal fractions of PB pretreated rats under anaerobic conditions

Halogenated Alkane	NADPH Oxidation (nmol/mg protein/min)	Inhibition by CO (%) *
CCl_3-CCl_3	35 (± 2)	99
CCl_3-CCl_2H	31 (± 3)	99
F_3C-CCl_3	9 (± 0.5)	81
CCl_4	2.7 (± 0.2)	81
F_3C-CHBrCl	0.8 (± 0.1)	98
$ClCH_2$-CH_2Cl	0	-
$CHCl_2$-$CHCl_2$	0	-
CCl_3-CH_3	0	-
CHCl-CCl_2	0	-

Each value is the mean ± S.D. from 3 experiments.
* Data are expressed as percentage of controls.

The metabolites of hexachloroethane with microsomes from PB pretreated rats were identified as the olefin tetrachloroethene (99.5%) and the alkane pentachloroethane (0.5%). From pentachloroethane the corresponding metabolites were identified as trichloroethene (96%) and tetrachloroethane (4%).

The rates of formation of these products in anaerobic NADPH reduced liver microsomes of rats after different pretreatments are listed in Table 2.

In liver microsomes from phenobarbitone-pretreated rats the production of tetrachloroethene (from C_2Cl_6) and trichloroethene (from C_2HCl_5) is strongly induced compared to control rats, whereas the formation of the saturated products of both halogenated alkanes was less affected. 3-Methylcholanthrene failed to increase the reductive metabolism of hexa- and pentachloroethane compared to control rats.

The rates of formation of products from both

Table 2. Effect of inducers on the reductive dehalogenation of hexa- and pentachloroethane

Pretreatment	Cytochrome P450 (nmol/mg protein)	Hexachloroethane C_2Cl_4 (nmol/mg protein/min.)	Hexachloroethane C_2HCl_5 (nmol/mg protein/min.)	Pentachloroethane C_2HCl_3 (nmol/mg protein/min.)	Pentachloroethane $C_2H_2Cl_4$ (nmol/mg protein/min.)
PB	2.2	27.0 ± 1.1	0.10 ± 0.01	28.5 ± 1.7	1.20 ± 0.04
3-MC	1.1	4.9 ± 0.8	0.06 ± 0.02	4.2 ± 0.5	0.54 ± 0.08
Control	0.75	8.0 ± 1.2	0.05 ± 0.01	6.4 ± 0.5	0.64 ± 0.07

Each value is the mean ± S.D. from 3 experiments.

Table 3. Comparison of the formation of products and the oxidation of NADPH in a preparation of liver microsomes from PB induced rats

Substrate	Products formed		NADPH oxidation
	Olefin	Alkane	
	(nmol/mg protein/min.)		(nmol/mg protein/min.)
C_2Cl_6	33.9 ± 5	0.2 ± 0.02	36.8 ± 5
C_2HCl_5	35.0 ± 3	0.8 ± 0.1	38.5 ± 1

Each value is the mean ± S.D. from 4 experiments.

hexa- and pentachloroethane were comparable to the rates of the NADPH oxidation. The average stoichiometry for several PB induced microsomal preparations ($n = 3$) was 1.07 ± 0.2 nmoles C_2Cl_4/nmole NADPH, and 1.12 ± 0.2 nmoles C_2HCl_3/nmole NADPH for hexa- and pentachloroethane, respectively. This suggested that two electrons are needed for the formation of one olefin molecule (Table 3).

In order to obtain further evidence that the reduction of the polyhalogenated alkanes was a cytochrome P450 dependent process, the product formation was studied under various conditions. The results of the large scale incubations of anaerobic reduced microsomal fractions with hexa- and pentachloroethane are summarized in Table 4.

Molecules combining with the catalytic site of cytochrome P450 such as CO and metyrapone inhibited the reductive dehalogenation of hexa- and pentachloroethane to a similar extent as was shown for carbon tetrachloride or halothane [19]. As in the case of halothane and carbon tetrachloride, high concentrations of metyrapone (1 mM) are necessary to obtain about 70 per cent inhibition of the reductive dehalogenation. The inhibition was significantly higher in microsomes of PB induced rats compared to 3-MC and controls due to the higher affinity of this inhibitor to the PB induced forms of cytochrome P450 [20].

Table 4. Effect of various inhibitors on the NADPH supported dehalogenation of hexachloroethane and pentachloroethane in liver microsomes of PB pretreated rats

Condition		Hexachloroethane		Pentachloroethane	
		C_2Cl_4	C_2HCl_5	C_2HCl_3	$C_2H_2Cl_4$
		(% inhibition)		(% inhibition)	
CO	10^{-3}M	98 ± 1	86 ± 7	96 ± 1	74 ± 5
Metyrapone	10^{-4}M	46 ± 10	41 ± 8	48 ± 4	32 ± 6
Metyrapone	10^{-3}M	66 ± 8	79 ± 10	67 ± 9	36 ± 6
α-Naphthoflavone	10^{-4}M*	13 ± 2	26 ± 4	-	-
Boiled microsomes		99 ± 1	95 ± 1	96 ± 1	74 ± 5
NADPH omitted		99 ± 1	99 ± 2	99 ± 1	87 ± 5

Each value is the mean ± S.D. from 4 experiments.
* α-Naphthoflavone was dissolved in a 0.1% albumin solution.

10^{-4}M Metyrapone inhibited the formation of the main products tetrachloroethene (from C_2Cl_6) and trichloroethene (from C_2HCl_5) by 47 ± 10% for PB and by 27 ± 6% for 3-MC and 22 ± 3% for controls. The inhibition by α-naphthoflavone was only 13 per cent for hexachloroethane in microsomes from PB induced rats compared to 44 ± 8 per cent for 3-MC (results not shown). Carbon monoxide inhibited the olefin formation by more than 95 per cent.

The rate of the NADH supported dehalogenation of hexachloroethane was 6 per cent of the NADPH dependent reaction (Table 5). After the addition of both nucleotides the formation of tetrachloroethene from hexachloroethane was higher than the sum of the NADPH and NADH supported reactions. This synergistic effect suggested that the second electron in the reduction process of these polyhalogenated alkanes can also be transferred via cytochrome b_5 to cytochrome P450 as it is known for the dioxygen-dependent monoxygenase reaction [21]. Correspondingly, the level of reduced cytochrome b_5, which is almost completely reduced under anaerobic conditions with NADPH as well as NADH as reducing agent, was decreased from 100 to 80 per cent upon addition of hexachloroethane and then increased from 80 to 85 per cent following the addition of NADH. The synergistic effect was essentially the same for pentachloroethane (results not shown).

Since the transfer of the second electron by either NADPH reductase or cytochrome b_5 can be influenced by pH [22, 23] the product pattern of the dehalogenation reaction of hexachloroethane was investigated at various pH-values in anaerobic liver microsomes from PB pretreated rats (Fig. 1).

NADPH plus NADH were used to ensure optimal reduction of the components of the microsomal monooxygenase system. The formation of the two electron reduction product tetrachloroethene is maximal at pH 7.6, whereas the production of the one electron reduction product pentachloroethane is increasing even up to pH 8.8. The ratio of C_2HCl_5/C_2Cl_4 over the pH range 7.2 to 8.8 shows that the one electron reduction product increased compared to the two electron reduction with increasing pH.

The influence of radical traps on the product pattern of the reductive dehalogenation of hexachloroethane has been investigated in liver microsomal incubations of rats pretreated with PB (Table 6). The production of pentachloroethane was increased by cysteine (10 mM) and penicillamine (1 and 10 mM) by about 100 per cent compared to controls; cysteine (1 mM) and cystine (1 mM) exhibited only a small increase and glutathione (10 mM) had no effect on the rate of formation. The production of tetrachloroethene was not changed by the sulfur compounds and, correspondingly, the rate of NADPH oxidation was not altered by inclusion of these thiols (results not shown).

The influence of dioxygen on the reductive dehalogenation of hexachloroethane at various dioxygen concentrations in liver microsomes of rats pretreated with PB is shown in Fig. 2.

The rate of reduction was between 80 and 40 per cent in the presence of 1 and 5 per cent dioxygen, respectively, compared to anaerobic incubations.

Table 5. The synergistic effect of NADH on the reductive dehalogenation of hexachloroethane in liver microsomes of PB induced rats

Conditions	C_2Cl_4 (nmol/mg protein/min.)	%	C_2HCl_5 (nmol/mg protein/min.)	%
NADPH 2×10^{-4}M	21.5 ± 1.0	(100)	0.06 ± 0.020	(100)
NADH 2×10^{-4}M	1.3 ± 0.2	(6)	0.01 ± 0.004	(20)
NADPH 2×10^{-4}M and NADH 2×10^{-4}M	29.4 ± 1.2	(137)	0.11 ± 0.014	(173)

Each value is the mean ± S.D. from $n = 4$ experiments. The NADPH generating system was replaced in these experiments by a direct addition of the pyridine nucleotides. The incubation time was 5 min.

This dioxygen concentration is assumed to be present in the central lobular region of the liver [24]. But also at high oxygen tension (about 20 to 100% dioxygen) there still exists 20 to 10 per cent of the dehalogenation rate, respectively, compared to anaerobic incubations. The ratio of the olefin to the alkane was not significantly affected by the presence of dioxygen.

DISCUSSION

Halogenated alkanes are lipophilic compounds and, as such, are able to interact with the active site of liver microsomal cytochrome P450. This was established for haloalkanes [25, 26] by measuring the formation of the high-spin enzyme–substrate complex in oxidised microsomal fractions. Under anaerobic conditions many polyhalogenated alkanes are reductively dehalogenated by NADPH in liver microsomal incubations. This reduction in most cases is mediated by cytochrome P450 [5, 13] as our results also indicate for hexa- and pentachloroethane.

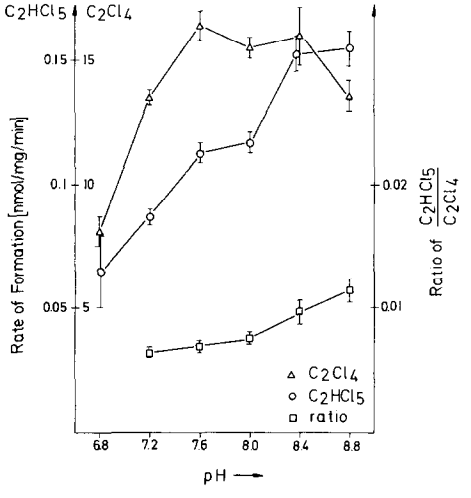


Fig. 1. pH-Dependence of the dehalogenation of hexachloroethane in anaerobic liver microsomes from PB pretreated rats in the presence of NADPH. Incubations under dinitrogen in 4 ml 50 mM HEPES buffer contained 150 mM KCl, 0.1 mM sodium azide, 4 mg microsomal protein (2.6 nmoles cytochrome P450/mg protein), 0.2 mM NADPH and 0.5 mM hexachloroethane. After incubation at 30° for 5 min, the reaction was stopped by addition of 0.4 ml $HClO_4$ (10% v/v), extracted with pentane (5 ml) and analyzed by g.l.c. Each value represents the mean ± S.D. from 3 experiments.

Of the compounds tested the best acceptors for the electrons of NADPH are hexa- and pentachloroethane. The low NADPH oxidation observed with 1,1,1-trifluorotrichloroethane, halothane and carbon tetrachloride may be due to the formation of a stable intermediate cytochrome P450 complex [12] which inhibits further reductive metabolism of these compounds or due to destruction of cytochrome P450 by reactive metabolites [27]. Accordingly, there is no indication for the existence of any stable intermediate complex of the reduced cytochrome P450 with hexachloro- and pentachloroethane and only a minor destruction of cytochrome P450 with these compounds.

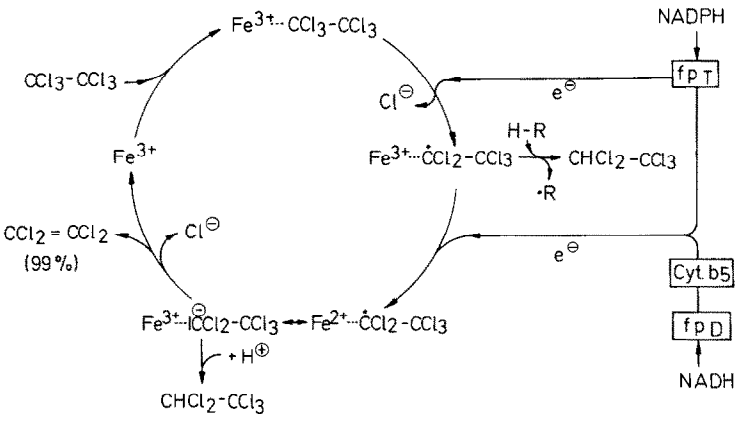
The inhibition of NADPH oxidation and product formation by CO, metyrapone and α-naphthoflavone demonstrates that the reductive metabolism of hexa- and pentachloroethane is cytochrome P450 dependent. Those isoenzymes induced by PB are most effective favouring especially the olefin formation from the haloethane, whereas the 3-MC induced forms have a much lower turnover for these compounds. This is in agreement with the results of other reports, showing, that the reductive metabolism of halogenated alkanes is increased after pretreatment of animals by PB [5, 13].

Hexa- and pentachloroethanes were dehalogenated mainly to the corresponding olefins tetra- and trichloroethene [28]. Their rates of formation were comparable to the rates of NADPH oxidation suggesting that two electrons are needed for the production of one molecule of product. For hexachloroethane this stoichiometry is compatible with the following equation:

$$NADPH + H^+ + CCl_3\text{–}CCl_3 \rightarrow NADP^+ + CCl_2{=}CCl_2 + 2HCl$$

Olefins have also been established as products of the reductive metabolism of halothane *in vitro* [13] and *in vivo* [29]. Their mechanism of formation should be analogous to the two electron reduction postulated for carbon tetrachloride [12] and halothane [13]. A mechanism for these reactions involving hexachloroethane is proposed in Scheme 1.

The one electron reduction of hexachloroethane by reduced cytochrome P450 under anaerobic conditions can lead to the radical ferric cytochrome



Scheme 1

complex ($Fe^{3+}\cdot CCl_2$–CCl_3). In contrast to the corresponding complexes that have been postulated for carbon tetrachloride and halothane [12, 13] this species does not seem to release the radical from the active site, since only a very small amount of pentachloroethane is formed by abstraction of a hydrogen atom from its environment by the free radical whereas in the known case of carbon tetrachloride 80 per cent of the ferric cytochrome radical complex ($Fe^{3+}\cdot CCl_3$) decay to chloroform [12].

The radical complex can accept a second electron from the cytochrome P450 to form the carbanion complex ($Fe^{3+}\ ^{-}ICCl_2$–CCl_3). Although this complex could be protonated to yield pentachloroethane this does not seem to occur, since a stoichiometric quantity of tetrachloroethene can be detected. Hence β-elimination must be the preferred reaction, in agreement with the chloride ion being a good leaving group for this process. In accordance with this assumption no carbene or carbanion complex as in the case of carbon tetrachloride or halothane, respectively, could be found.

The proposed two electron reduction mechanism is supported by the results of the pH dependence of the reductive dehalogenation of hexachloroethane. According to Werringloer and Kawano [22] and Noshiro *et al.* [23] the reactions associated with the transfer of the first and second electron are controlled differentially in an opposing pH dependent manner.

Table 6. Effect of various sulfur compounds on the dehalogenation of hexachloroethane by NADPH reduced liver microsomes of PB pretreated rats

S-H-Compounds		Tetrachloroethene (% of control)	Pentachloroethane (% of control)
Cysteine	1 mM	101 ± 1	132 ± 11
Cysteine	10 mM	103 ± 16	201 ± 11
Cystine	1 mM	106 ± 12	117 ± 23
Penicillamine	1 mM	115 ± 20	211 ± 56
Penicillamine	10 mM	129 ± 8	236 ± 49
Red. glutathione	1 mM	108 ± 10	103 ± 7
Red. glutathione	10 mM	108 ± 9	97 ± 14

Each value is the mean ± S.D. from 3 experiments.

These effects are also seen for the reductive dehalogenation of hexachloroethane supposed that the β-elimination and hydrogen abstraction are not dependent on the pH. Thus, at high pH where the facility for the first reduction step is optimal compared to the second reduction step the ratio of pentachloroethane to tetrachloroethene is increased.

In contrast at low pH where the second reduction step is facilitated compared to the first reduction step the ratio of the product pattern is shifted to the olefin as the two electron reduction product. A partial transfer of the second electron via cytochrome b_5 is also supported by the distinct synergistic effect seen in the presence of both NADPH and NADH. The synergistic effect can be explained by an increased level of reduced cytochrome b_5 thus favouring the supply of electrons to cytochrome P450 and the halogenated compound [23].

The postulated radical species formed in this reaction cycle is not quenched by radical trapping agents. This is suggested by the unchanged rate of

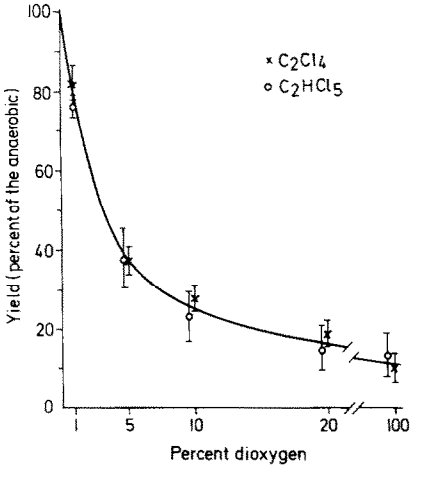


Fig. 2. Effect of dioxygen on the dehalogenation of hexachloroethane by liver microsomes of PB pretreated rats in the presence of NADPH. Assays as described in Materials and Methods. Value represents the mean ± S.D. (n = 3).

olefin formation for which the cytochrome P450 radical complex is the precursor. The increase of pentachloroethane may be explained by the inhibition of covalent binding of the free radical to membrane components leading to more alkane product. The same effect is seen also in the reductive dehalogenation of carbon tetrachloride in the presence of radical traps [12, 30].

Like carbon tetrachloride hexachloroethane cannot be metabolized oxidatively due to its lack of any hydroxylatable C–H bonds. Hence only the reductive pathway can occur which is confirmed by the exhalation of tetrachloroethene and pentachloroethane in sheep after application of hexachloroethane [31, 32]. Both metabolites, however, may explain the known diversity of metabolites found in sheep after pretreatment with hexachloroethane.

For pentachloroethane one can also postulate an oxidative pathway leading to trichloroacetic acid [33]. This is in complete analogy to the formation of trifluoroacetic acid from halothane [34]. The extent by which oxidative and reductive metabolism occurs with these two compounds must be a function of the dioxygen concentration which is the physiological substrate for reduced cytochrome P450 and therefore must block the reductive pathways competitively. This was confirmed in our study also for hexachloroethane, but like in the case of carbon tetrachloride [12] or halothane [13] a remarkably high concentration of dioxygen is required for this inhibition. Under a physiological oxygen tension of about 4 per cent [24] the reduction rate is still around 50 per cent of that in the completely anaerobic system.

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