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THE MECHANISM OF REDUCTIVE DEHALOGENATION OF HALOTHANE BY LIVER CYTOCHROME P450*

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Abstract—The reductive dehalogenation of halothane leading to 2-chloro-1,1,1-trifluoroethane (CTE) and 2-chloro-1,1-difluoroethylene (CDE) has been investigated *in vitro* using rat liver microsomes under anaerobic conditions. The stimulation of NADPH oxidation by halothane as well as the formation of the products were dependent upon cytochrome P450 as indicated by their CO and metyrapone inhibition. After replacement of NADPH by sodium dithionite as a reducing agent CDE was the only product of the enzymatic reaction. The product pattern was influenced by pretreatment with 3-methylcholanthrene, benzo(a)pyrene, phenobarbitone and Arochlor 1254 and by addition of anti-cytochrome P450-PB immunoglobulin. The CTE:CDE ratio was shifted by addition or inhibition of cytochrome b_5 and by pH variation indicating a crucial role of the second electron donation to cytochrome P450 in determining the product pattern. The intermediate complex of cytochrome P450 with a Soret band at 470 nm formed with halothane in reduced liver microsomes was shown to decompose spontaneously to give CDE. Therefore we propose the 470 nm peak to represent a cytochrome P450 Fe^{3+}---$^{\ominus}$$CHCl-CF_3$ carbanion complex. From these results a reaction pathway could be derived which includes radical and carbanion intermediates as reactive precursors of CTE and CDE, respectively.

The widely used inhalation anaesthetic halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) is metabolized in man to a considerable extent [1]. Its major metabolite, trifluoroacetic acid, clearly is a product of a monooxygenase pathway [2] which proved to be a detoxication reaction, whereas some toxic actions of halothane have been reported to correlate with a reductive pathway [3]. Hypoxic conditions favour the reductive metabolism *in vivo* which is paralleled by an increased covalent binding of labelled halothane with decreasing dioxygen tension *in vitro* [4]. The covalently bound metabolites still contained the labelled chlorine and ^3H [5, 6] indicating that not monooxygenation but rather a reductive elimination of bromine had formed the reactive metabolite. This agrees with a lack of an effect of deuteration on the toxicity, although the oxidative metabolism was inhibited [7].

The covalent binding under anaerobic conditions is paralleled by a release of fluoride *in vitro* [8], which suggested a common pathway for fluoride splitting and toxicity. *In vivo* experiments also suggested a correlation between fluoride release and hepatotoxicity [3].

Recently two volatile metabolites of halothane, 2-chloro-1,1,1-trifluoroethane (CTE) and 2-chloro-1,1-difluoroethylene (CDE), have been identified *in vivo* in rabbit [9] and in man [10]. Their formation is also favoured by hypoxic conditions and by phenobarbitone (PB) induction [11]. Several mechanisms have been proposed to explain the formation of CDE and CTE as well as the covalent binding to cellular constituents under hypoxic conditions involving one or two electron reductions leading to radicals, carbanions [10] and carbenes [12] as reactive intermediates. Since there is now direct evidence that many polyhalogenated alkanes can undergo reductive dehalogenation by microsomal cytochrome P450 [13, 14] and owing to the severe implications for the halothane toxicity *in vivo* it seemed worthwhile to clarify the mechanism of this reaction in a well defined *in vitro* system under anaerobic conditions.

Therefore, this study was undertaken to show that microsomal cytochrome P450 catalyses the reductive dehalogenation of halothane, that the product pattern is dependent on the composition of the monooxygenase system and that the reduction of halothane results from consecutive one electron transfers analogous to the previously reported reduction of carbon tetrachloride [14]. An attempt was also made to elucidate the nature of the metabolic intermediate complex of cytochrome P450 and halothane characterised by an absorption maximum at 470 nm in the difference spectrum [12]. A preliminary report of some of this work has been made [15].

MATERIALS AND METHODS

Male Sprague–Dawley rats (100–150 g) were used after treatment with sodium phenobarbitone (PB) (80 mg/kg body weight (b. wt) i.p., daily for 3 days), Arochlor 1254 (500 mg/kg b. wt i.p., a single dose 4 days prior to use), 3-methylcholanthrene (3-MC) (20 mg/kg b. wt i.p. daily for 2 days) and benzo(a)pyrene (20 mg/kg b. wt i.p., for two days). Liver microsomal fractions were prepared by the method of Frommer *et al.* [16]. Protein content was determined by the biuret method [17], cytochrome

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P450 by the procedure of Omura and Sato [18] and the b_5 content by the method of Strittmatter [19].

Purified cytochrome b_5 free of detergents and phospholipids was obtained from pig liver microsomes by the method of Strittmatter [19] and kindly supplied by Dr. J. Poensgen. The antibodies against b_5 from pig were elicited in young male rabbits and purified by ammonium sulfate fractionation and DE-52 column chromatography [20] and were a gift by Dr. H. Graf. Antibodies against cytochrome P450 were prepared by Dr. M. Noshiro.

Halothane (Fluothane®) was purchased from ICI-Pharma, Plankstadt, Federal Republic of Germany. 2-Chloro-1,1,1-trifluoroethane and 2-chloro-1,1-difluoroethylene were obtained from Fluorochem Ltd, Glossop, UK. All other chemical compounds and standard reagents were obtained from regular commercial sources.

The halothane induced NADPH oxidation was measured in rat liver microsomal preparations (1 mg protein/ml) suspended in 0.1 M Tris–HCl buffer pH 7.6 in stoppered 1 cm glass cuvettes. Dioxygen was removed by bubbling the buffer for 10 min with dinitrogen prior to the addition of microsomes followed by bubbling another 5 min with N_2. After addition of NADPH (100 μM) and halothane (1 mM) to the test cuvette the absorption change at 340 nm was recorded with time against a reference containing microsomes in an Aminco-DW2 spectrophotometer in the split beam mode at 37°. An extinction coefficient of 6.22 mM^{-1} cm^{-1} was used for calculating the rate of NADPH oxidation.

The assay for volatile halothane metabolites was performed in 10 ml screw capped vials containing 12 mg of microsomal protein in 2.5 ml 0.1 M Tris–HCl buffer pH 7.6, unless specified otherwise, and a NADPH generating system (glucose-6-phosphate (16 mM), $MgCl_2$ (12 mM), glucose-6-phosphate dehydrogenase (3 U), $NADP^+$ (0.5 mM)).

Dioxygen was removed by bubbling the buffer for 10 min with N_2, then adding the microsomal suspension and bubbling for another 5 min. The reaction was started by addition of halothane (1 mM). After 15 min incubation at 37°, 0.2 ml of 10% perchloric acid was added and head space analysis of the volatile metabolites was carried out according to Maiorino *et al.* [21] on a HP 5700 gas chromatograph equipped with FID.

For incorporation of cytochrome b_5 the microsomal suspension in 0.25 M sucrose containing 20 mM Hepes (pH 7.5) and 0.1 mM EDTA at a protein concentration of 30 mg/ml was preincubated for 15 min at room temperature with pure cytochrome b_5 free from detergents and phospholipids according to Noshiro *et al.* [22]. This b_5 fortified microsomal suspension was used directly in the assay for volatile halothane metabolites.

The incubations with antibodies were performed by adding 2.5 mg immunoglobulin to the buffer in the assay for halothane metabolites described above prior to the addition of 0.5 mg microsomal protein. The anaerobic suspension was then preincubated for 30 min at room temperature before starting the reaction.

The formation of volatile halothane metabolites in the presence of dioxygen was measured after addition of increasing amounts of dioxygen via a gas-tight syringe to the anaerobic assay described above. Sufficient equilibration was guaranteed by shaking thoroughly during incubation.

For the measurement of the metabolic intermediate complex of halothane, microsomal fractions were suspended in 2.5 ml of anaerobic 0.1 M Tris–HCl buffer pH 7.6. Incubations were performed at 30° in stoppered 1 cm glass cuvettes and contained microsomal protein (2 mg/ml) and NADPH (0.2 mM) or sodium dithionite (5 mM). The reaction was started by addition of halothane (1 mM) to the test cuvette and the formation of the complex was monitored in an Aminco DW2 spectrophotometer in the split beam mode for 2 min. CO was added by bubbling the test cuvette for 10 sec and scans were taken over 15 min before addition of potassium ferricyanide (to a final concentration of 10 mM) and re-reduction with sodium dithionite (2 mg).

RESULTS

The anaerobic oxidation of NADPH by microsomes in the presence of halothane can be considered as an appropriate measure of the reductive metabolism of halothane. Figure 1 shows such a typical experiment.

The rate of oxidation was not linear with time so that only the total consumption after 15 min can be given. As an average value for microsomes from control rats 6.9 ± 2.1 nmoles mg $protein^{-1}$ was found. Phenobarbitone pretreatment increased this value to 15.1 ± 1.7 nmoles mg $protein^{-1}$ and Arochlor 1254 pretreatment even raised it to 24 ± 2 nmoles mg $protein^{-1}$. NADPH-oxidation was inhibited by carbon monoxide (1 mM) to 95% and by metyrapone (1 mM) to 65% whereas reduced glutathione had no effect.

In these incubations two volatile metabolites of

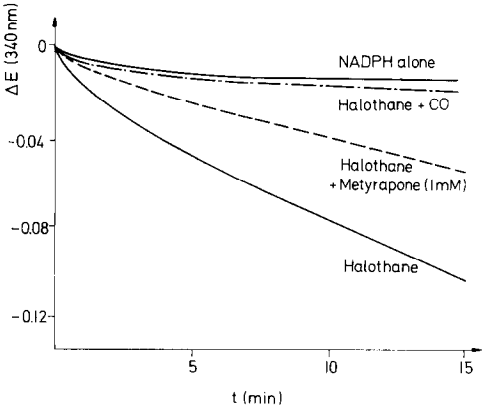


Fig. 1. Effect of halothane on the rate of NADPH oxidation in anaerobic liver microsomal preparations from PB pretreated rats. The NADPH oxidation was measured by monitoring the decrease in absorption at 340 nm at 37° as described in Materials and Methods. CO was added by bubbling the incubation medium with CO for 30 sec prior to the addition of halothane (1 mM). Metyrapone was added in Tris–HCl buffer to a final concentration of 1 mM. The protein concentration was 1.0 mg/ml containing 1.7 nmoles cytochrome P450/mg protein.

Table 1. Effect of different inducing agents on the formation of volatile halothane metabolites in anaerobic NADPH reduced rat liver microsomes

Pretreatment	Cyt. P450 (nmol/mg)	Cyt. b_5 (nmol/mg)	Products formed (nmol/mg/15 min)		Product ratio
			CDE	CTE	CTE/CDE
PB	2,25 ± 0.31	0.67 ± 0.04	3.6 ± 1.0	10.0 ± 1.0	2.93 ± 0.68
Arochlor	2.85 ± 0.64	0.78 ± 0.02	2.6 ± 1.1	6.9 ± 1.1	2.70 ± 0.30
3-MC	1.24 ± 0.19	0.73 ± 0.05	2.1 ± 0.4	4.3 ± 0.6	1.92 ± 0.39
BP	1.55	0.69	3.0 ± 0.4	5.3 ± 0.9	1.76 ± 0.09
None	0.82 ± 0.18	0.55 ± 0.04	1.9 ± 0.4	3.4 ± 0.6	1.88 ± 0.04

The assays were performed as described in Materials and Methods. The values represent means ± S.D. from at least 3 different preparations except benzo(a)pyrene (mean ± S.D. of 3 exp.).

halothane, 2-chloro-1,1,1-trifluoroethane (CTE) and 2-chloro-1,1-difluoroethylene (CDE) could be detected by head space gas chromatography. NADH as electon donor was only 20–30 per cent as active as NADPH and both nucleotides together exerted an additive but not a synergistic effect (results not shown).

Since it is known that the microsomal monooxygenase system consists of multiple forms of cytochrome P450, which vary in amount by pretreatment with inducing drugs, the two reductive metabolites were determined after pretreatment of rats with different inducers.

As a result two groups could be distinguished. Pretreatment with 3-methylcholanthrene (3-MC) and benzo(a)pyrene caused only a small and balanced increase of CDE and CTE compared to untreated animals, whereas phenobarbitone (PB) and Arochlor 1254 pretreatment increased the yield mainly by increasing CTE, so that the CTE/CDE ratio markedly increased (Table 1).

Table 2. Effect of various inhibitors on the NADPH supported formation of volatile halothane metabolites in anaerobic, PB induced rat liver microsomal fractions

Conditions	CDE	CTE
	(% of uninhibited reactions)	
Air saturated	0.1 ± 0.05	0.2 ± 0.03
CO saturated	2 ± 1	6 ± 1
Metyrapone (10^{-4}M)	48 ± 6	52 ± 11
NADPH omitted	3 ± 1	1 ± 0.2
Boiled microsomes	4 ± 1	1 ± 0.2
Red. glutathione (10^{-2}M)	94 ± 7	74 ± 8
Oxid. glutathione (10^{-2}M)	99 ± 5	64 ± 6

The assays were performed as described in Materials and Methods. The various inhibitors were present in the incubation medium before starting the reaction, CO was bubbled for 30 sec in the anaerobic suspension. Cytochrome P450 content was 2.3 ± 0.3 nmoles/mg protein. The values represent means ± S.D. from 3 different microsomal preparations.

That both metabolites were formed reductively by cytochrome P450 forms could be concluded from inhibition studies with carbon monoxide and metyrapone (Table 2).

Reduced and oxidised glutathione, known to interact with radicals, significantly decreased the yield of CTE, whereas CDE was not affected. Sodium dithionite can effectively reduce cytochrome P450 and guarantees complete anaerobiosis of the microsomal suspension. This artificial electron donor gave absolute yields of CDE of 4.5 ± 0.5 nmoles mg protein^{-1} 15 min^{-1} and of CTE of 7.5 ± 2.2 nmoles mg protein^{-1} 15 min^{-1}. Therefore the relative amount of CTE was less than in the NADPH supported reaction. CO and metyrapone proved to be inhibitory for CDE formation but CTE production was not influenced (Table 3).

Since boiled microsomes were equally effective with dithionite, CTE clearly was formed by a non-P450-dependent reaction. The time course for CDE formation was slightly higher with dithionite than with NADPH and less inhibited with time (Fig. 2). The rate of CTE formation was very high in the

Table 3. Effect of various inhibitors on the dithionite supported formation of volatile halothane metabolites in PB induced rat liver microsomal fractions

Conditions	CDE	CTE
	(% of uninhibited reactions)	
CO saturated	6 ± 3	100 ± 21
Metyrapone (10^{-4}M)	58 ± 8	117 ± 20
Dithionite omitted	3 ± 1	1 ± 0.3
Boiled microsomes	3 ± 2	118 ± 46

The assays for volatile halothane metabolites were performed as described in Materials and Methods. Sodium dithionite (5 mM) was added instead of NADPH generating system and the reaction was stopped additional to $HClO_4$ by $K_3Fe(CN)_6$ (10 mM final concentration). Cytochrome P450 content was 2.2 ± 0.2 nmoles/mg protein. The values represent means ± S.D. from 3 different microsomal preparations.

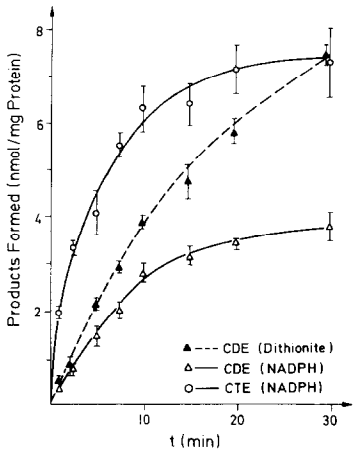


Fig. 2. Formation with time of volatile halothane metabolites in anaerobic reduced rat liver microsomes. The assays were performed at 37° as described in Materials and Methods. For the measurement of the dithionite supported reaction, the NADPH generating system was replaced by sodium dithionite (5 mM final concentration) and the reaction was stopped by addition of 0.2 ml 10% $HClO_4$ and 10 mM $K_3Fe(CN)_6$. The cytochrome P450 content was 2.2 nmoles/mg protein.

initial phase and declined rapidly with time suggesting a turnover-dependent inhibition of the enzyme.

Due to the high affinity of cytochrome P450 for dioxygen it could be expected that the reductive metabolism of halothane was suppressed by dioxygen in the medium. However, even in the presence of 0.5% dioxygen still about 50 per cent of CTE and CDE were formed with CDE being more suppressed than CTE (Fig. 3).

As will be pointed out in the discussion, the mechanism of CTE and CDE formation occurs quite analogously to the formation of the $\cdot O_2H$ radical and the oxenoid complex when dioxygen is reduced by cytochrome P450 in two subsequent one electron steps [23]. Since under certain conditions cytochrome b_5 can contribute to the donation of the second electron to the oxycomplex of cytochrome-P450 [22, 24] we attempted to influence the CTE/CDE ratio by either blocking cytochrome b_5 with its antibody or by incorporating cytochrome b_5 into microsomes, in order to block or to stimulate the electron transfer to a hypothetical cytochrome P450 halothane radical complex.

Figure 4 shows that b_5 incorporation into microsomes increases the yield of CDE without affecting greatly that of CTE. In accordance with the results on the monooxygenase activity this effect was higher at high pH. The corresponding CTE/CDE ratio reached low values coming close to those of control microsomes.

A pronounced dependence of the ratio was also found by varying the pH. The yield of CDE was steadily decreasing with increasing pH whereas CTE formation had an optimum at pH 7.6 (Fig. 5).

This caused an increase of the ratio at higher pH values, suggesting that the supply of the second electron required for CDE formation was less favourable at higher pH.

A complementary experiment has been performed by inhibiting the cytochrome b_5 dependent electron transfer by a specific anti-b_5 immunoglobulin (Table 4).

The amount of anti-b_5 immunoglobulin added was sufficient to inhibit the NADH dependent cytochrome c reduction by 95 per cent. This amount decreased the CDE formation significantly by 25 per cent ($P < 0.01$) and increased the CTE production slightly. An antibody against the main form in PB pretreated rat liver microsomes depressed preferentially CTE production, indicating that this form

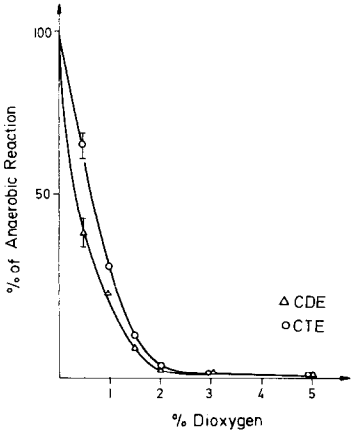


Fig. 3. Influence of dioxygen on the formation of volatile halothane metabolites in anaerobic PB induced rat liver microsomes. The assays were performed as described in Materials and Methods but at room temperature under vigorous shaking. Increasing amounts of dioxygen were introduced by a gas-tight syringe. The cytochrome P450 content was 1.7 nmoles/mg protein.

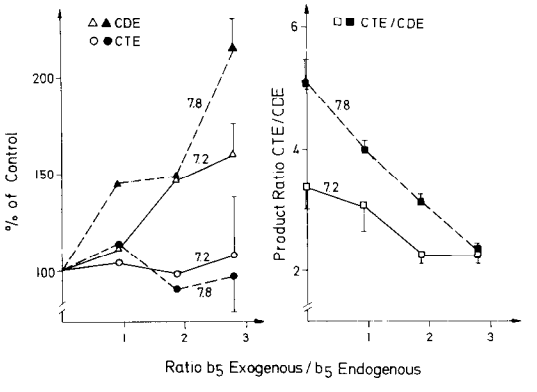


Fig. 4. Influence of b_5 incorporation on the yields of the volatile halothane metabolites and on the product ratio CTE/CDE in liver microsomes from PB pretreated rats. Assays were perfomed in 2.5 ml of 50 mM Hepes buffer pH 7.2 (——) and pH 7.8 (- - -) containing 0.1 mM EDTA and 12 mg microsomal protein (2.6 nmoles cytochrome P450/mg and 0.61 nmoles cytochrome b_5/mg), preincubated with various amounts of purified cytochrome b_5. After removing dioxygen NADPH (0.2 mM) and NADH (0.2 mM) were added and the reaction performed as described under Materials and Methods.

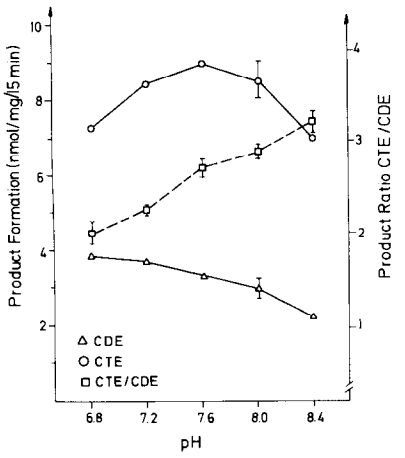


Fig. 5. Influence of pH on the rates of formation of volatile halothane metabolites in anaerobic PB induced rat liver microsomes. Assays were performed in buffers containing 50 mM Hepes at various pH values, 150 mM KCl, 0.1 mM EDTA and 12 mg microsomal protein (2.9 nmoles cytochrome P450/mg). The NADPH generating system was replaced by NADPH (0.5 mM) and NADH (0.2 mM). The procedure of the assays was identical to that described under Materials and Methods.

of cytochrome P450 produced mainly CTE instead of CDE.

The reductive metabolism of halothane is accompanied by spectral changes occurring at cytochrome P450 with the development of an absorption band at 470 nm in the difference spectrum. NADPH or dithionite may serve as reductants [12]. Although this spectral species was assigned by previous work from our laboratory as a ferrous P450–carbene complex [12], it could also represent an intermediate responsible for either the formation of CTE or CDE.

An approach to that problem seemed possible by quenching the reaction in the steady state. This does not affect the 470 nm complex immediately, but first led to a superimposed CO spectrum (Fig. 6).

Table 4. Influence of antibodies on the product pattern of the reductive halothane dehalogenation in anaerobic PB induced rat liver microsomes

Conditions	CDE	CTE	Product ratio
	(nmol/mg/15 min)		CTE/CDE
No IG	2.3 ± 0.1	9.2 ± 1.4	4.0 ± 0.4
	(100 %)	(100 %)	
Control IG	2.4 ± 0.2	9.4 ± 2.1	3.7 ± 0.6
	(104 %)	(102 %)	
Anti b_5 IG	1.7 ± 0.2	10.2 ± 1.8	6.0 ± 0.5
	(74 %)	(111 %)	
Anti P450 IG	1.9	6.3 ± 0.5	3.3
	(82 %)	(68 %)	

The assays were performed as described in Materials and Methods. Cytochrome P450 content was 1.9 nmoles/mg protein and the cytochrome b_5 content 0.67 nmole/mg. The values represent means ± S.D. ($n = 3$). Significance was tested by Student's t test ($P < 0.01$).

The shoulder at 470 nm then slowly decreased with time accompanied by an increase of the absorption at 450 nm indicating a slow decomposition of the intermediate complex and a formation of free cytochrome P450. Addition of potassium ferricyanide as oxidant immediately destroyed the complex and after rereduction the full cytochrome P450 CO spectrum could be observed.

This experiment allows the estimate that about 50 per cent of the total cytochrome P450 is present at the intermediate 470 nm complex under fully reduced conditions. For NADPH reduced anaerobic liver microsomes from PB-pretreated animals the fraction of blocked cytochrome P450 was essentially the same. The millimolar extinction coefficient for the metabolic intermediate complex in the difference spectrum between 470 nm and 510 nm could be calculated as $80 \pm 4\ mM^{-1}\ cm^{-1}$.

The amount of complex in dithionite reduced microsomal fractions as well as its stability against decomposition under CO was strongly dependent upon pretreatment of the animals (Table 5).

In microsomes from PB and Arochlor pretreated animals the steady-state level of the metabolic intermediate complex was higher than in microsomes from controls or benzo(a)pyrene or 3-MC induced rats. Since it was noted that the conversion of the 470 nm band to the CO complex proceeded slower in microsomes from PB and Arochlor pretreated animals, it seemed probable that the 470 nm complex represented the steady-state of a P450 halothane intermediate which then decomposed to one of the products. We therefore investigated the influence of CO on the yields of products as shown in Table 6.

It can be seen that before formation of the complex an almost complete and uniform inhibition of both metabolites was observed. When CO was added after completion of the complex formation only CTE was inhibited to the same extent but CDE was still formed. It therefore seemed as if the complex decomposed to give CDE. If microsomes from controls were used, where the 470 nm complex was very small, the difference of CO inhibition before and after complex formation was not significant.

Assuming that 0.4 mmole/l CO could block CDE formation from halothane completely, one could design an experiment which would allow to calculate the amount of CDE derived from relaxation from the steady-state (Table 7).

In this experiment the 470 nm complex was allowed to build up during 2 min and then the reaction was quenched by CO and allowed to proceed for 15 min. Subtracting the control values under CO for 15 min and the uninhibited reaction for 2 min, one obtained a total yield of CDE which must have been formed during exposure to CO. This value was beyond the experimental error and suggested that CDE was formed by decomposition of the steady state level of the 470 nm complex. This was supported by the amount of P450 recovered during 15 min as measured by the formation of new P450 CO complex under the same conditions. From this we conclude that CDE is a product of the intermediate 470 nm complex.

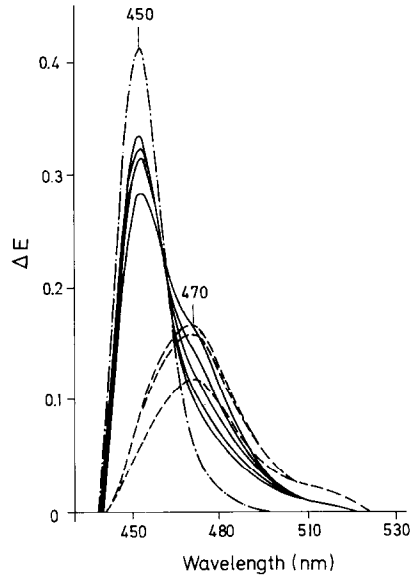


Fig. 6. Influence of CO on the metabolic intermediate complex of halothane in sodium dithionite reduced liver microsomal fractions from PB pretreated rats. Incubations in stoppered 1 cm cuvettes were performed as described in Materials and Methods. The protein concentration was 2 mg/ml with a cytochrome P450 content of 2.3 nmoles/mg protein. --- Sodium dithionite (5 mM) and halothane (1 mM) added. Scans taken at 0.5, 1.0 and 2.0 min. —— CO bubbled for 10 sec. Scans taken at 0.5, 5, 10 and 15 min. -·- Oxidised by addition of potassium ferricyanide (10 mM) re-reduced with an excess of sodium dithionite and bubbled with CO for 20 sec.

DISCUSSION

It was the main purpose of our investigation of the reductive metabolism of halothane to fit also this compound to our previously suggested general scheme for the reductive mechanism of dehalogenation of polyhalogenated compounds [25]. There is no doubt that halothane, like carbon tetrachloride [14] or hexachloroethane [25], is a substrate for a cytochrome P450 dependent reduction process in which NADPH and NADH are the physiological electron donors and dithionite can serve as an artificial one. The basis of our general concept is, that cytochrome P450 can bind the lipophilic polyhalogenated compounds to form an enzyme–substrate complex which then is reduced by one electron in the absence of dioxygen. The reduced ferrous cytochrome would donate its electron to the substrate and lead to a radical after releasing a halogenide anion. In the case of halothane the bromide would be the most likely candidate and the resulting radical could either form a complex with the ferric cytochrome or could be released from the active site. In the case of carbon tetrachloride the $\cdot CCl_3$ radical forms chloroform by an abstraction process since

Table 5. Effect of different pretreatments on the formation of the metabolic intermediate complex of halothane

Pretreatment	Cyt. P450 (nmol/mg Pr.)	Cyt. P450 complexed (%)	Δ E (/mg/ml)	Stability
PB	2.25 ± 0.31	52 ± 2	0.05 ± 0.01	high
Arochlor	2.85 ± 0.64	54 ± 14	0.06 ± 0.01	high
3-MC	1.24 ± 0.19	19 ± 8	0.02 ± 0.005	low
BP	1.55	15 ± 4	0.01 ± 0.001	low
None	0.82 ± 0.18	4 ± 1	0.01 ± 0.001	low

Values were taken from spectroscopic experiments performed as described in Materials and Methods. The values represent means ± S.D. from at least 3 different microsomal preparations except benzo(a)pyrene (mean ± S.D. of 3 exp.).

Table 6. Effect of the metabolic intermediate complex of halothane on the CO inhibition of the reductive dehalogenation of halothane in NADPH and NADH reduced liver microsomes

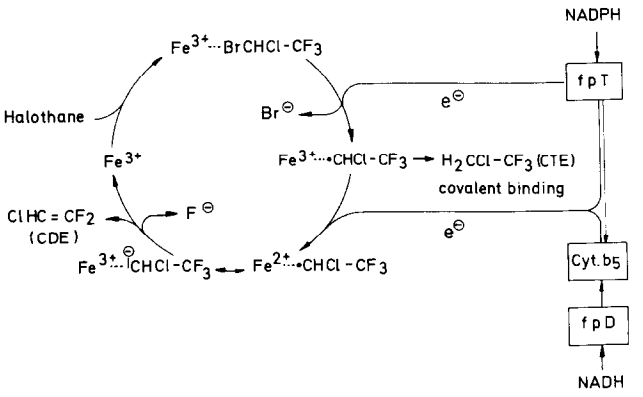
CO inhibition	CDE (% inhibition)	CTE (% inhibition)	Pretreatment
Before formation of the complex	96 ± 3	92 ± 2	PB
After formation of the complex	68 ± 2	94 ± 6	PB
Before formation of the complex	98 ± 1	95 ± 2	None
After formation of the complex	91 ± 6	98 ± 4	None

The assays for the volatile halothane metabolites were performed as described in Materials and Methods. The cytochrome P450 content was 2.8 nmoles/mg for PB and 0.9 nmole/mg for controls. NADPH (0.5 mM) and NADH (0.2 mM) were added before starting the reaction with halothane (1 mM). The complex formation was inhibited by addition of 1 ml Tris–HCl buffer pH 7.6 saturated with CO before starting and the formation of the complex was allowed by 2 min preincubation with halothane before adding the CO solution (1 ml). Inhibition was calculated relatively to a 15 min incubation without CO and to a 15 min incubation after subtraction of the products from the preincubation, respectively. The values represent means ± S.D. ($n = 4$).

Table 7. Formation of CDE following the destruction of the metabolic intermediate complex of halothane in dithionite reduced liver microsomes from PB pretreated rats

	Incubation time Preincubation	+ CO	CDE formation (nmol/mg)	Cyt. P450 recovered in 15 min (nmol/mg)
(1)	2 min	15 min	1.30 ± 0.04	
(2)	-	15 min	0.16 ± 0.06	
(3)	2 min	-	0.51 ± 0.03	
	(1) - (2) - (3)		0.63 ± 0.13	0.59 ± 0.10

The assays for CDE were performed as described in the legend for Table 6 with sodium dithionite (5 mM) as reducing agent. Cytochrome P450 content was 2.28 nmoles/mg. The cytochrome P450 recovered in 15 min was obtained from experiments performed as described in the legend of Fig. 6 by a two component analysis of the spectral changes at 450 nm and 470 nm after addition of CO to the halothane intermediate complex. The values represent means ± S.D. ($n = 4$) of a typical experiment.



Scheme 1. Proposed mechanism of the reductive dehalogenation of halothane. Fe^{2+}, Fe^{3+} represent the heme center of cytochrome P450, fpT the NADPH dependent cytochrome P450 reductase and fpD the NADH dependent cytochrome b_5 reductase.

little deuterochloroform was detected in ^2H_2O [14]. A corresponding experiment was also performed with halothane [26] and the lack of incorporation of deuterium is indicative of the same mechanism for halothane reduction to CTE.

A free ·CHCl–CF_3 radical should also be able to bind covalently to microsomal proteins and unsaturated lipids. Correspondingly the covalently bound metabolites of halothane contain ^{36}Cl [5] and ^3H [6] which further supports the hypothesis of a free ·CHCl–CF_3 as a precursor of CTE.

In the presence of NADPH or dithionite the radical complex can accept a second electron to form a cytochrome P450 Fe^{3+}---$^{\ominus}$CHCl–CF_3 complex (Scheme 1).

This anion complex with a σ-bond could then release fluoride via β-elimination to form CDE. This would occur in complete analogy to the formation of tetrachloroethylene from hexachloroethane [25]. In principle, the anion complex could also release chloride to form a carbene complex as postulated from previous experiments [12]. In addition, it could add a proton to yield CTE but this pathway does not seem to operate since no deuterium from ^2H_2O was found to be incorporated in CTE [26].

From our results an assignment can be made with regard to the nature of the 470 nm complex. It converts to CDE in a stoichiometric amount, suggesting that it may be the carbanion complex of CTE (Fe^{3+} CTE^- or Fe^{2+} ·CTE). From the postulated carbene complex this would not be possible. The decomposition of the 470 nm complex is not followed by a formation of CTE and the rate of formation of CTE is maximal when the 470 nm complex is not yet formed which argues against the ferric CTE radical complex (Fe^{3+} ·CTE) being the 470 nm species. Preliminary experiments indicate indeed that the 470 nm complex is associated with a low spin ferric heme, whereas in contrast the ferrous P450 dichlorocarbene complex (Fe^{2+} $^1CCl_2$) did not show a signal. The definite structure of this ferric heme species remains to be solved by means of model complexes.

It is an interesting feature of our mechanistic scheme that it resembles closely the mechanism of oxygen activation by cytochrome P450 [23]. The Fe^{3+} ·CTE radical complex would then be formally analogous to the oxy-complex of P450, which also releases its radical partially to give $\cdot O_2^-$ and H_2O_2 [27]. The donation of a second electron leads to a Fe^{3+} peroxide species equivalent the Fe^{3+} CTE^- carbanion complex. Both can undergo elimination of OH^- or a fluoride, respectively, to give the oxenoid complex $[FeO]^{3+}$ or CDE.

The analogy fits even closely with regard to the electron transport system. During the hydroxylation of many substrates, the second electron can be donated partly by cytochrome b_5 in microsomes from phenobarbitone pretreated rats [22]. This is a pH-dependent reaction which is fast at low pH-values and not effective at high pH [24]. Addition of cytochrome b_5 to the microsomes causes a decrease of the cytochrome P450 oxycomplex and an increase of the monooxygenase activity indicating a more effective turnover of dioxygen to the hydroxylating species [22]. Correspondingly, cytochrome b_5 addition to a reconstituted system decreases O_2^- formation and increases the monooxygenation reaction by a more rapid reduction of the oxy complex [28]. In our experiments, addition of cytochrome b_5 decreases the CTE/CDE ratio whereas the antibody against b_5 increased it significantly. The yield of CTE was not affected indicating that CDE but not CTE requires a second electron for its formation. In the presence of dithionite the radical of CTE may be trapped effectively by either ferrous cytochrome P450 or dithionite itself, so that only CDE is formed. The shift in the CTE/CDE ratio with pH can easily be explained by the different rates of first to second electron transfer and, also, the varying yields of CTE and CDE after different pretreatments may be a consequence of differences in the electron transport rates and/or differences in the stability of the intermediates.

Since cytochrome b_5 is not much enhanced by the inducing agents used, the relative amount of b_5 compared to cytochrome P450 is low in microsomes from PB and Arochlor pretreated rats and, correspondingly, the CTE/CDE ratio is high. Addition of b_5 to microsomes from PB pretreated rats up to a relative amount of b_5 near to that found in controls, 3-MC

and BP treated animals decreases the CTE/CDE ratio near to that of controls.

Thus the CTE/CDE ratio under anaerobic conditions can be used as a parameter of electron transport similar to the H_2O_2/monooxygenation product ratio for the aerobic monooxygenase pathway. That dioxygen addition affects the yield of CDE more than of CTE may be due to a more rapid oxidation of the Fe^{3+} carbanion complex compared to the ·CTE radical complex which chemically must be expected.

Besides the interesting implications for the mechanism of microsomal electron transport and for the coordination chemistry of cytochrome P450, our findings and the postulated reaction scheme also help in clarifying the toxic events associated with the reduction of halothane and other polyhalogenated hydrocarbons. The amount and the reactivity of the radicals formed during reductive dehalogenation of haloalkanes seem to be two of the determinants for hepatotoxicity. Compared to halothane, carbon tetrachloride is a potent hepatotoxin and indeed gives high amounts of radicals during reduction [14]. In agreement with our finding that CTE indicates also a radical formation during reductive dehalogenation halothane causes an increased exhalation of ethane or pentane after *in vivo* administration which is considered to be indicative of lipid peroxidation due to metabolic radical formation [29]. Also the retention of Cl and H from halothane in the covalently bound metabolites points to the involvement of the radical as the attacking species [5, 6]. The amount of volatile products recovered from the reductive dehalogenation of halothane was always smaller than the total NADPH consumption and this was particularly evident after PB and Arochlor pretreatment. This also indicates that a considerable portion of the total turnover leads to covalent binding, probably due to radical attack. In agreement with this assumption we found higher levels of CTE, the reaction product of the radical, in microsomes from PB and Arochlor pretreated animals. *In vivo* studies with these inducers had revealed a marked increase of halothane hepatotoxicity [3, 30]. Under completely anaerobic conditions the 470 nm complex seems to decompose entirely to CDE and intact cytochrome P450. It is not known at present what effect small concentrations of dioxygen would have on this complex. If it autoxidizes, reactive peroxides of CTE may be formed which could contribute to the binding to cell constituents. However, the influence of dioxygen on the reductive pathway is complex and needs further investigation.

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