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The Reduction of Polyhalogenated Methanes by Liver Microsomal Cytochrome P450

C. ROLAND WOLF,[1] DANIEL MANSUY,[2] WOLFGANG NASTAINCZYK, GENEVIÈVE DEUTSCHMANN, AND VOLKER ULLRICH

Department of Physiological Chemistry, University of the Saarland, 665 Homburg, Saar, German Federal Republic

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SUMMARY

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Under anaerobic reducing conditions various polyhalogenated methanes (CBr_4, CCl_4, CCl_3F, CCl_3Br, CCl_3CN, CHI_3, $CHBr_3$, and $CHCl_3$) form complexes with ferrous cytochrome P450 with absorption peaks in the difference spectra ranging between 450 and 470 nm. Carbon monoxide was detected as a metabolic product of the interaction. Inhibition experiments indicated the involvement of cytochrome P450 in the reduction process, and a reaction sequence is proposed to account for these findings. Comparison of cytochrome P450 complex formation using liver microsomal preparations from phenobarbital- and 3,4-benzpyrene-treated rats showed differences which could be accounted for by decreased stability of the halogenomethane complex with the 3,4-benzpyrene-induced form of cytochrome P450.

INTRODUCTION

Many polyhalogenated aliphatic hydrocarbons undergo chemical reactions *in vivo* which result in increased toxicity rather than in detoxification (1). Carbon tetrachloride has been investigated extensively in this respect, but the mechanism of its hepatotoxicity has not yet been completely elucidated. It has been suggested that it is not an oxidative but a reductive process, via chloride elimination and radical formation, which leads to covalent binding to cell constituents and cell necrosis (2). Treatment of experimental animals with phenobarbital greatly enhances carbon tetrachloride toxicity (3-5), which suggests that cytochrome P450 is involved in this reductive activation. This cytochrome is involved in the reduction of molecular oxygen for monooxygenase reactions (6) and is also known to reduce organic nitro and azo compounds (7, 8). Experiments *in vitro* with liver microsomes have shown that an NADPH-dependent reduction of carbon tetrachloride to chloroform occurs which requires anaerobic conditions and can be inhibited by carbon monoxide (9).

It has also been reported that under anaerobic reducing conditions carbon tetrachloride forms a complex with cytochrome P450 with a Soret absorption band at 454 nm in the difference spectrum (10). This

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[1] Recipient of financial support from the Royal Society.

[2] Present address, Laboratoire de l'Ecole Supérieure, 75231 Paris, France.

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band was tentatively assigned by us to a ligand complex of the reduced heme iron with the trichloro carbanion, which could release a chloride ion yielding the dichloro carbene (11). Chemical evidence for such a reductive elimination of halogens by reduced cytochrome P450 was later found to occur with halothane. Under reducing conditions a complex with reduced cytochrome P450 was formed which could also be obtained with 1,1,1-trifluorodiazoethane, suggesting the corresponding carbene as the ligand (12). We wished to learn whether this could be a general metabolic pathway for polyhalogenated hydrocarbons and therefore investigated the spectral interactions of a series of halogenated methane derivatives with rat liver microsomes. Some preliminary experiments have already been reported (13).

MATERIALS AND METHODS

All chemicals used were obtained from commercially available sources.

Male Sprague-Dawley rats (100–150 g) treated with sodium phenobarbital (80 mg/kg of body weight, one intraperitoneal injection per day for 3 days) or 3,4-benzpyrene (20 mg/kg of body weight, injected intraperitoneally as a solution in olive oil daily for 2 days) were used. Liver microsomal fractions were prepared according to Frommer *et al.* (14). Protein was determined by the biuret method (15), and cytochrome P450, by the method of Omura and Sato (16). Rat hemoglobin was prepared according to Rossi-Fanelli and Antonini (17) and was used as an aqueous solution.

The carbon monoxide formed during anaerobic microsomal incubations was determined by measurement of the carbon monoxide–hemoglobin spectrum. Quantitative values for carbon monoxide concentrations were obtained from calibration curves determined by titration of a sodium dithionite-reduced microsomal suspension containing hemoglobin (25 μM) with microliter quantities of an aqueous solution saturated with carbon monoxide. Microsomes were suspended in 0.1 M Tris-HCl buffer, pH 7.6; final volume, 3 ml, 1 mg of microsomal protein per milliliter. The difference absorption between 419 nm and the isosbestic point at 425 nm was measured in 1-cm glass cuvettes, using an Aminco DW-2 spectrophotometer, and was plotted against carbon monoxide concentration. The carbon monoxide concentration of a saturated aqueous solution was taken as 1.0 mM at 20° (18).

Substrate-induced difference spectra were measured in liver microsomal preparations suspended in 0.1 M Tris-HCl buffer, pH 7.6, reduced with sodium dithionite or NADPH. Spectra were recorded in stoppered 1-cm glass cuvettes containing 3 ml of microsomal suspension (1 mg of protein per milliliter), using an Aminco DW-2 spectrophotometer. In experiments under anaerobic conditions involving NADPH, the microsomes were first bubbled for 10 min with nitrogen, followed by the addition of substrate (4 mM) and NADPH (100 μM).

CCl_4-induced NADPH oxidation under anaerobic conditions was measured using the experimental procedure described above. The rate of NADPH oxidation was measured by monitoring the absorption difference between 340 and 500 nm. The effect of carbon monoxide on the CCl_4-induced rate of NADPH oxidation was determined by bubbling the microsomal suspension for 30 sec with carbon monoxide before the addition of NADPH. The CCl_4-induced rate of NADPH oxidation was also determined in the presence of metyrapone (50 mM) added prior to the addition of substrate and NADPH. Quantitative values for the rate of NADPH oxidation were obtained using an extinction coefficient of 6.2 $mM^{-1}\ cm^{-1}$.

Simultaneous measurements of oxygen concentration and formation of the substrate–ferrous cytochrome P450 complex were made using a platinum-silver Clark oxygen electrode fitted into a 1-cm glass cuvette, as described by Nastainczyk and Ullrich (19).

RESULTS

Many halogenated alkanes are known to give typical type I difference spectra when added to liver microsomal suspensions (20), representing the formation of an enzyme-substrate complex. All the com-

pounds used here exhibited similar spectra.

Figure 1 shows the typical difference spectrum obtained after incubation of CCl_4 with sodium dithionite-reduced liver microsomal suspensions from phenobarbital-treated rats. The formation of the spectrum was time-dependent, and a gradual shift in the position of the Soret band from 460 to 454 nm was observed. On the addition of hemoglobin to test and reference cuvettes after peak formation, the λ_{max} shifted back to 460 nm, and a typical hemoglobin–carbon monoxide difference spectrum was obtained at 419 nm.

In incubations with CBr_4, CCl_3F, CCl_3Br, CCl_3CN, $CHBr_3$, $CHCl_3$, or CHI_3 similar difference spectra were formed, and in most cases a concomitant shift in the Soret band was recorded (Table 1). In the case of CCl_3CN and $CHCl_3$ no shift was observed. In contrast to the other compounds, the $CHCl_3$ complex formed very slowly, 30–40 min being required before a maximum value was obtained. The magnitude of the spectrum obtained with CCl_3Br was much smaller than for the other compounds tested and was only 20% of the CCl_4 spectrum.

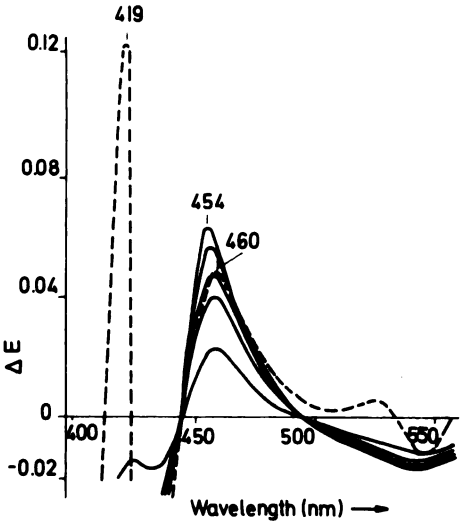


FIG. 1. *Difference spectrum obtained after addition of CCl_4 to sodium dithionite-reduced liver microsomal preparations*

The two cuvettes contained 3 mg of microsomal protein (2.1 nmoles of cytochrome P450 per milligram) in 3.0 ml of 0.1 M Tris-HCl buffer, pH 7.6. After reduction with sodium dithionite (2 mg), CCl_4 (4 mM) was added to the test cuvette. The scans shown were taken at 0.3, 1.0, 1.7, 4.4, and 10.0 min. - - -, difference spectrum obtained after the addition of hemoglobin to test and reference cuvettes after 10 min of incubation.

Inclusion of hemoglobin in the incubation medium inhibited the shift in the Soret band (Table 1), and a time-dependent increase in the hemoglobin–carbon monoxide spectrum was observed. With CCl_3CN or $CHCl_3$, no significant carbon monoxide–hemoglobin spectrum was obtained. These results indicated that the shift in the absorption band was due to the formation of carbon monoxide during the incubation. The inhibition of the shift by hemoglobin can be explained by its high affinity for carbon monoxide, which prevents carbon monoxide from binding to cytochrome P450.

The presence of carbon monoxide was confirmed by drawing the gases dissolved in a microsomal suspension, after incubation in the presence of CCl_4 and sodium dithionite, into a gas cuvette (path length, 10 m) inside a Perkin-Elmer infrared spectrophotometer, model 325. The resultant spectrum showed bands typical of carbon monoxide (Fig. 2). Control experiments in

TABLE 1

λ_{max} values obtained on interaction of various halogenomethanes with ferrous cytochrome P450 in the presence of sodium dithionite

Experimental details were the same as in Fig. 1.

Compound	Substrate–cytochrome P450 complex:		
	0.3 min	20 min	Hemoglobin[a]
	nm	*nm*	*nm*
CCl_4	460	454	459
CBr_4	465	455	465
CCl_3F	453	452	453
CCl_3Br	454	453	(455)
CCl_3CN	468	468	468
$CHCl_3$	464	464	464
$CHBr_3$	465	455	464
CHI_3	465	463	464
CH_2Cl_2	No complex formed		
CH_2Br_2	No complex formed		
CH_3Cl	No complex formed		

[a] Experiments carried out after addition of hemoglobin (25 μM) to test and reference cuvettes.

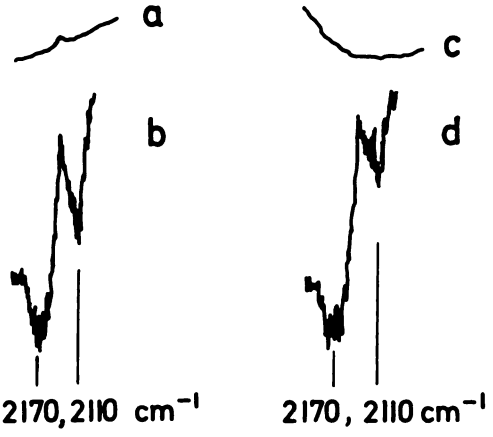


FIG. 2. *Comparison of infrared spectrum of pure carbon monoxide gas with that of gases dissolved in a sodium dithionite reduced microsomal suspension after incubation with CCl_4*

Each milliliter of incubation medium, in 0.1 M Tris-HCl buffer, pH 7.6, contained microsomal protein (2 mg), sodium dithionite (2 mg), and CCl_4 (4 mM) in a total volume of 100 ml. Incubations were carried out in stoppered two-necked flasks. After 40 min of incubation at 30°, the dissolved gases were drawn into an evacuated gas cuvette inside a Perkin-Elmer (model 325) spectrometer, and the spectra were recorded. *a*, baseline; *b*, pure carbon monoxide gas (approximately 2500 nmoles); *c*, incubation in the absence of CCl_4; *d*, incubation in the presence of CCl_4.

the absence of CCl_4 and with acid-denatured microsomes gave no spectrum in the region scanned.

The rates of carbon monoxide formation for the various compounds tested are shown in Fig. 3. Carbon monoxide was first detectable after about 1 min of incubation, whereas the ferrous cytochrome P450 complex was immediately visible. The initial rates of formation of the cytochrome P450 complexes were unaffected by the presence of hemoglobin. These findings indicate that the spectrum observed initially was due solely to halogenomethane complex formation.

CH_3Cl, CH_2Cl_2, and CH_2Br_2 did not lead to carbon monoxide formation under anaerobic conditions, and also no spectral change could be observed with these halogenated methanes, as noted in Table 1.

Similar results for all halogenomethanes investigated were obtained when NADPH was substituted for sodium dithionite as reductant. Cytochrome P450 complex formation and concomitant formation of carbon monoxide were observed. The rates of both carbon monoxide and complex formation were comparable. In the presence of metyrapone, 80% inhibition of both complex and carbon monoxide production was recorded. No interaction was measurable using resuspended, acid-denatured microsomes.

To obtain further evidence that the interaction of the halogenomethanes was NADPH- and cytochrome P450-dependent, the substrate-induced rate of NADPH oxidation under anaerobic conditions was investigated. Figure 4 shows the results obtained after the addition of CCl_4 to anaerobic liver microsomal preparations. The increase in rate of NADPH oxidation measured after 1 min was 3.4 nmoles/min/mg of protein. In the presence of carbon monoxide and metyrapone the rates were 0.4 and 0.7 nmole/min/mg of protein, respectively, which represents 88% inhibition by carbon monoxide and 79% by metyrapone.

The direct involvement of cytochrome P450 in complex and carbon monoxide formation was established using cytochrome P450 purified from phenobarbital-induced

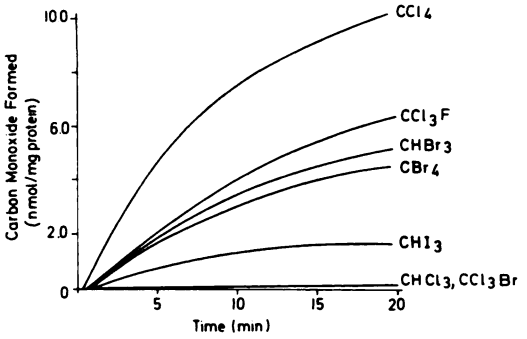


FIG. 3. *Rate of carbon monoxide formation after addition of various halogenomethanes to sodium dithionite-reduced liver microsomal preparations from phenobarbital-treated rats*

Each incubation contained 3.0 mg of microsomal protein suspended in 3 ml of 0.1 M Tris-HCl buffer, pH 7.6, hemoglobin (25 μM), sodium dithionite (2 mg), and substrate (4 mM). The spectral difference between 419 and 425 nm was scanned with time. Absolute values for carbon monoxide concentration were obtained from a calibration curve.

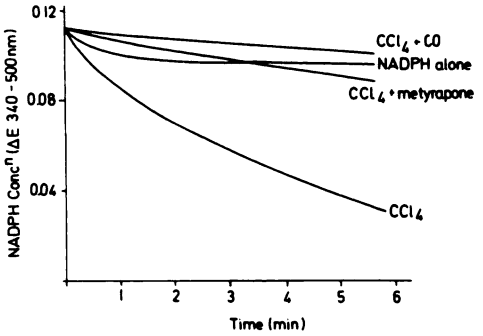


FIG. 4. *Effect of CCl_4 on rate of NADPH oxidation in anaerobic rat liver microsomal preparations from phenobarbital-treated rats*

Microsomal protein (3 mg) was suspended (3 ml) in 0.1 M Tris-HCl buffer, pH 7.6, containing 33 μM NADPH. NADPH oxidation was measured in the presence and absence of CCl_4 (4 mM) in anaerobic cuvettes by monitoring the absorption difference between 340 and 500 nm. The effect of carbon monoxide on the CCl_4-induced rate was determined by bubbling the incubation medium with carbon monoxide for 30 sec before the addition of NADPH. Experiments were also performed in the presence of 50 mM metyrapone.

rabbit liver microsomes by the method of Duppel *et al.* (21). The cytochrome P450 preparation contained 12 nmoles/mg of protein and was free of NADPH–cytochrome P450 reductase and cytochrome b_5. On addition of CCl_4 to the sodium dithionite-reduced cytochrome, the substrate–cytochrome P450 complex formed and a shift in the position of the Soret band from 460 to 454 nm was recorded with time. In the presence of hemoglobin no shfit in λ_{max} was observed, and the concomitant formation of carbon monoxide was measured.

The formation of the halogenomethane–cytochrome P450 complexes was also determined at low oxygen pressure. Figure 5 shows the results of a typical experiment using CCl_3F. In this case the Soret band started to form at approximately 5% oxygen. In the case of CCl_4 the spectrum formed at an oxygen concentration of 8%. Similar experiments also showed that carbon monoxide was not formed at high oxygen concentrations with CCl_4 and CCl_3F, which suggests that complex and carbon monoxide formation are mediated by the same reaction pathway.

In microsomal preparations from rats treated with 3,4-benzpyrene the halogenomethanes also formed complexes with ferrous cytochrome P450, with Soret bands in the region of 450 nm. However, peak heights, based on cytochrome P450 content, tended to be smaller than the equivalent peaks using microsomes from phenobarbital-treated animals, and formed more slowly. In the presence of hemoglobin no shift in the Soret band was observed, and the magnitude of the spectra formed was greatly reduced. Figure 6a and b compares the spectra of CCl_4 obtained in the presence and absence of hemoglobin. In this case the spectrum was 60% smaller in the presence of hemoglobin. Figure 6b shows that time-dependent formation of carbon monoxide also occurred (confirmed by infrared spectroscopy). Table 2 compares the magnitude of the halogenomethane–cytochrome P450 complex and the rate of carbon monoxide formation, using microsomes from phenobarbital- or 3,4-benzpyrene-treated rats. It can be seen that 3,4-benzpyrene-induced microsomes led to higher rates of carbon monoxide formation, accompanied by smaller ΔE_{max} values of the corresponding cytochrome P450 complexes.

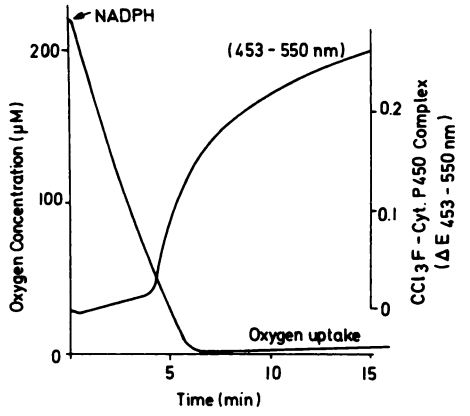


FIG. 5. *Simultaneous monitoring of oxygen uptake and formation of CCl_3F-ferrous cytochrome P450 complex*

Incubations were carried out at 25°, using liver microsomal preparations suspended in 0.1 M Tris-HCl buffer, pH 7.6, containing 4 mg/ml of microsomal protein and 4 mM CCl_3F. The reaction was started by the addition of NADPH (1 mM), and the difference absorption between 453 and 550 nm was scanned in parallel with oxygen uptake.

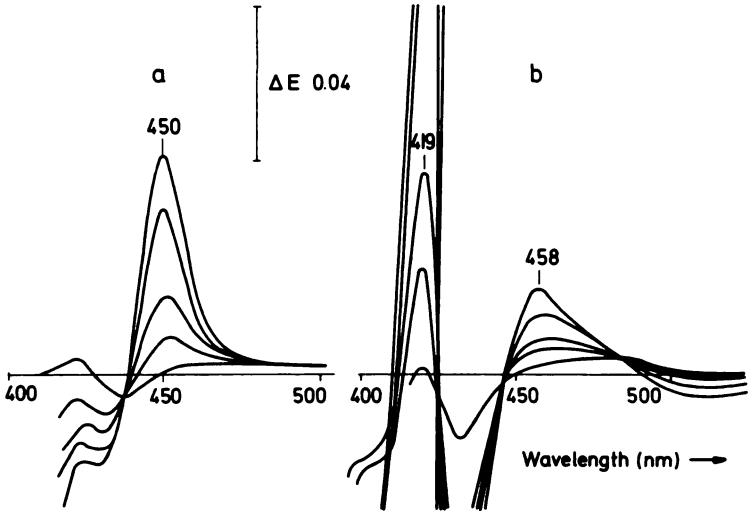


FIG. 6. *Measurement of CCl_4-ferrous cytochrome P450 complex in liver microsomal preparations from 3,4-benzpyrene-treated rats, reduced with sodium dithionite in the presence (b) and absence (a) of hemoglobin*

The cytochrome P450 concentration was 1.8 nmoles/mg of protein. In experiments involving hemoglobin, hemoglobin (25 μM) was added to test and reference cuvettes. Other experimental details were the same as in Fig. 1. The scans shown were taken at 0.3, 0.8, 1.5, 4.5, and 10.0 min.

TABLE 2

Comparison of halogenomethane interactions in liver microsomes from phenobarbital- and 3,4-benzpyrene-treated rats in the presence of sodium dithionite

Experiments to determine ΔE_{max} were carried out in the presence of 25 μM hemoglobin. Other experimental details were the same as in Figs. 1 and 3.

Substrate	$\Delta E\lambda_{max-500\ nm}$/nmoles cytochrome P450		Rate of carbon monoxide formation	
	PB[a]	BP	PB	BP
	nmoles/min/nmole P450			
CCl_4	0.028	0.011	0.48	1.2
CBr_4	0.019	0.012	0.20	0.70
CCl_3F	0.030	0.010	0.15	0.20
CCl_3CN	0.021	0.015	0	0
$CHCl_3$	0.013	0	Trace	Trace
$CHBr_3$	0.017	0.005	0.15	0.23
CHI_3	0.022	0.011	0.08	0.11

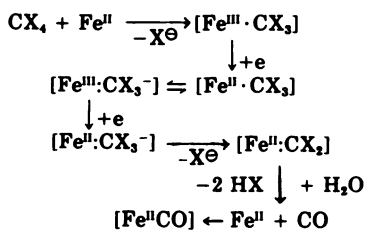
[a] PB, phenobarbital-treated; BP, 3,4-benzpyrene-treated.

DISCUSSION

All halogenated methane derivatives are lipophilic compounds and, as such, are able to interact with the active site of liver microsomal cytochrome P450. This can be seen from the spectra with oxidized microsomes, which all show the formation of the high-spin enzyme-substrate complex. Upon reduction under anaerobic conditions either with sodium dithionite or enzymatically with NADPH, various halogenomethanes exhibited different behavior. Those containing three or four halogens produced difference spectra with Soret bands between 450 and 470 nm, whereas dichloromethane and dibromomethane as well as the monohalogenated methanes gave no difference spectra. It is likely that the Soret bands represent low-spin ligand complexes of cytochrome P450. Such ligands appear to be formed by a reductive process from the corresponding tri- or tetrahalogenated methanes. This is supported by the increased rate of NADPH oxidation in microsomes upon addition of carbon tetrachloride, whereas the mono- and disubstituted methanes did not stimulate NADPH oxidation. The marked inhibition of metyrapone indicates that the reduction occurs at cytochrome P450.

The interpretation of the difference spectra observed was complicated by the finding that some of the observed peaks were not stable with time but increased, with a concomitant shift of the maxima. It was shown that this was due to formation

of the complex of cytochrome P450 with carbon monoxide, which was unequivocally identified by infrared spectroscopy. The evolution of carbon monoxide following a lag phase suggested that it may derive from the ligand complex. Prior reductive metabolism of the polyhalogenated methanes to a carbene ligand provides a feasible explanation:



This sequence of reactions can explain the experimental data and would be in agreement with the evidence for carbene complex formation using halothane. Carbon monoxide is a known hydrolysis product of dihalogeno carbenes (22, 23). That no carbon monoxide was formed in the case of CCl_3CN can be explained by the difficulty of hydrolyzing the corresponding carbene complex. Reports (24–28) concerning the interaction of transition metal complexes with polyhalogenated compounds tend to confirm the proposed reaction sequence. For example, CCl_4 is reduced by Cr^{II} complexes to give CO in high yields (24, 25). Various other di-, tri-, or tetrahalogenated methanes are also reduced by these complexes, the rate of reduction being proportional to the number of halogen substituents (24). A mechanism involving Cr^{II}-carbene complexes was proposed because the corresponding carbene could be transferred to an olefin, resulting in a cyclopropane derivative (25). Moreover, Fe^{II}-porphyrins and hemoproteins have been reported to reduce several halogenated compounds (26, 27). The reaction of Fe^{II}-deuteroporphyrin IX dimethyl ester with CCl_4 leads to a stable complex whose precise structure has not been determined, but which was postulated to be either CCl_3 or a $:CCl_2$ carbene complex (28).

The formation of the halogenomethane–cytochrome P450 complex at low oxygen concentration is an indication that these compounds compete with oxygen for the electrons of cytochrome P450, or, alternatively, that in the presence of oxygen the reaction mechanism differs.

When difference spectra were taken in the presence of sufficient amounts of deoxyhemoglobin to trap the carbon monoxide, only the halogenomethane complexes of cytochrome P450 could be observed.

The CCl_4-induced rate of NADPH oxidation was approximately 3 times faster than the measured rate of carbon monoxide formation. In the absence of oxygen, uncoupling reactions, reported for some cytochrome P450 monooxygenation reactions (29), cannot occur, and it seems probable that all the electrons from NADPH are transferred to the substrate. If carbon monoxide were the sole product of the interaction, 1:1 stoichiometry of NADPH to carbon monoxide would be expected. That this was not the case indicates that in this incubation system other reactions occur. Under similar incubation conditions CCl_4 and CCl_3F have been shown to give $CHCl_3$ and $CHCl_2F$, respectively, (9, 30); also, active metabolites of both these substrates bind irreversibly to microsomal proteins and lipids (31).[3]

It was interesting to find that carbon monoxide formation in microsomes from 3,4-benzpyrene-induced rats was higher than that from phenobarbital-treated rats. As the steady-state concentration of the carbene complex showed the reverse behavior, it could be argued that the benzpyrene-induced form of cytochrome P450 does not stabilize the carbene as well. This, in fact, has been found to be the case for many other ligand complexes (32, 33).

The question remains whether carbene complex formation is implicated in any way in the well-known hepatotoxicity of the polyhalogenated methanes. Certainly there are indications that the reduction of these compounds is a prerequisite to their toxicity. However, the toxic effects seem to be mediated mainly by the radical formed in the first reduction step, possibly leading to lipid peroxidation and subsequent destruction of cell membranes. Although a carbene intermediate could be formed in

[3] C. R. Wolf, T. Werner, L. J. King, and H. Uehleke, manuscript in preparation.

liver cells at low oxygen concentrations, it does not seem to be involved in the toxic reactions, as both CCl_4 and CCl_3F form carbene complexes but only CCl_4 initiates lipid peroxidation and is hepatotoxic (34).

Irrespective of its physiological significance, the possible formation of carbene complexes at cytochrome P450 is a fascinating aspect of this cytochrome which warrants further investigation.

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