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THE MECHANISM OF CHLOROFORM AND CARBON MONOXIDE FORMATION FROM CARBON TETRACHLORIDE BY MICROSOMAL CYTOCHROME P-450*

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Abstract—[^{14}C]carbon monoxide was a product of the anaerobic incubation of $^{14}CCl_4$ with hepatic microsomes and either NADPH or sodium dithionite. The reaction was dependent upon cytochrome P-450 as indicated by its CO and metyrapone inhibition and by the absence of any product after conversion of cytochrome P-450 to cytochrome P-420. The major metabolite produced in the NADPH-supported reaction was chloroform which, according to isotope experiments, seems to be mainly formed by hydrogen abstraction of the $\cdot CCl_3$ radical. The rate of CCl_4-induced NADPH-oxidation could be fully accounted for by the rate of formation of CO, $CHCl_3$ and covalently bound metabolites. Spectral studies indicated that the immediate precursor of the CO is probably the cytochrome P-450–Fe^{2+} dichlorocarbene complex. This was supported by the hydrolytic cleavage of an iron (II)-protoporphyrin IX dichlorocarbene complex to carbon monoxide. A reaction pathway for the formation of CO has been proposed including the possible radical, carbanion and carbene intermediates.

It is now well established that for hepatotoxicity to occur with CCl_4, prior metabolism involving cytochrome P-450 is essential. This aspect has been thoroughly reviewed by Recknagel and Glende [1]. The lipid solubility of the tetrahalomethanes marks them as potential substrates for the cytochrome P-450-dependent microsomal monooxygenase system but as they do not contain a C–H bond hydroxylation by direct oxygen insertion is not possible. There is ample evidence, however, demonstrating that under aerobic conditions CO_2 is a major metabolite of CCl_4 both *in vivo* [2–4] and *in vitro* [2, 5, 6]. Since direct hydroxylation cannot occur, the first stage in the metabolism of CCl_4 appears to be purely reductive resulting in the formation of the trichloromethyl radical, $CCl_3\cdot$, which has been confirmed by trapping with phenyl-tert-butyl nitrone [7, 8]. Under aerobic conditions this radical initiates lipoperoxidative damage *in vivo* and *in vitro* (reviewed in Ref. 1).

As the initial stage of CCl_4 metabolism is reductive, this aspect has been addressed *in vitro* using liver microsomal fractions under anaerobic conditions with NADPH as reducing agent. Under these conditions, the major pathway of CCl_4 metabolism results in the formation of chloroform accompanied by a covalent binding of $^{14}CCl_4$ carbon [9, 10]. It has also been reported that CCl_4 under anaerobic conditions forms a complex with cytochrome P-450 having a Soret absorption band at 454 nm [10]. A small amount of carbon monoxide was produced under these conditions using either NADPH or dithionite as reducing agents [11]. In this work evidence was presented that the 454 nm peak was a composite of a primarily formed 460 nm peak and the subsequent formation of a 450 nm peak which was identified as the cytochrome P-450–carbon monoxide complex.

The present studies with CCl_4 were undertaken to confirm that carbon monoxide is a true metabolite of CCl_4 under anaerobic, reducing conditions *in vitro*. This would primarily be of theoretical interest in view of the dichlorocarbene species as postulated intermediate and its analogy to the mechanism of dioxygen reduction and activation [12]. A preliminary report of some of this work has been made [13].

MATERIALS AND METHODS

Male Sprague–Dawley rats (100–150 g) were used after treatment with sodium phenobarbitone (80 mg/kg body wt) by intraperitoneal injection daily for 3 days. Liver microsomal fractions were prepared by the method of Frommer *et al.* [14]. Protein content was determined by the biuret method [15] and cytochrome P-450 by the procedure of Omura and Sato [16]. Rat haemoglobin was prepared from whole blood by the method of Rossi-Fanelli and Antonini [17] and was used as an aqueous solution. The covalent binding of $^{14}CCl_4$ to microsomal lipids and proteins was measured according to the method of Uehleke *et al.* [18]. $^{14}CCl_4$ was purchased from the Radiochemical Centre, Amersham, U.K. All other chemical compounds and standard reagents were obtained from regular commercial sources.

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Incubations with $^{14}CCl_4$. Incubations in 0.1 M Tris–HCl buffer, pH 7.6 (30 ml) contained microsomal protein (3 mg/ml), sodium dithionite (25 mg) or NADPH (1 mM) and 0.9 μCi $^{14}CCl_4$ (1 mM) in 0.1 ml methanol. Dioxygen was removed from the system by repeated evacuation and flushing with dinitrogen before introduction of the substrate. The mixture was incubated at 30° for 1 hr in a closed vessel, cooled (0°) and carrier ^{12}CO (3 μmoles) and conc. H_2SO_4 (5 ml) added. Volatile products were displaced by bubbling with dinitrogen (12 ml/min) for 5 hr and subsequent passing through a cold-trap of solid carbon dioxide–methanol to collect water vapour, followed by a column (20 × 2 cm) of heat-activated Kieselgel G to retain unreacted $^{14}CCl_4$ and the metabolite $^{14}CHCl_3$ and a catalytic column (6 × 0.5 cm) containing Hopcalite to oxidize ^{14}CO to $^{14}CO_2$. The $^{14}CO_2$ was absorbed in 20 ml phenylethylamine–methanol (1 : 1 v/v) contained in a tube packed with glass beads to aid absorption. Preliminary experiments with additional trapping tubes of phenylethylamine in series demonstrated that all the radioactivity was retained in the first tube throughout the 5 hr displacement with dinitrogen. The radioactivity in the phenylethylamine trap was determined by β-scintillation counting in a Packard Tri-Carb C 2425 using a toluene-based scintillant and internal standardization. Similar incubations were carried out for the possible formation of ^{14}C-formic acid or other non-volatile, water-soluble metabolites.

The anaerobic incubation were performed in 0.1 M Tris–HCl buffer, pH 7.6 (5 ml) containing microsomal protein (3 mg/ml), NADPH (1 mM) and 0.15 μCi $^{14}CCl_4$ (1.2 mM) added in 20 μl methanol. After incubating for 1 hr at 30°, the protein was denatured by heating in a boiling waterbath for 10 min and saturated sodium formate solution (1 ml) and 10 M NaOH (0.2 ml) were added. The aqueous supernatant fraction obtained after centrifugation was extracted with pentane (3 × 2 ml) and chloroform (3 × 2 ml) to remove lipid-soluble compounds. Then the aqueous phase was bubbled with dinitrogen (3 hr) to displace volatile products and residual organic solvent. Radioactivity remaining in the aqueous phase after neutralization by H_2SO_4 was determined by β-scintillation counting using Quickszint 454 (Zinsser, Frankfurt, F.R.G.).

Incubations with non-radioactive carbon tetrachloride. Microsomal fractions were suspended in 0.1 M Tris–HCl buffer, pH 7.6. The incubations of 2.5 ml were performed in 1 cm glass cuvettes at 30° and contained microsomal protein (1 mg/ml), haemoglobin (4 μM), carbon tetrachloride (1 mM) added in 10 μl methanol and sodium dithionite (approx. 2 mg). Carbon monoxide production was monitored by the difference absorption between the carboxyhaemoglobin peak at 419 nm and the isosbestic point at 411 nm in an Aminco DW-2 spectrophotometer in the dual beam mode. Quantitation was achieved by calibration curves constructed from values obtained by serial addition of microlitre quantities of saturated aqueous carbon monoxide solution (1.0 mM at 20°) to incubation mixtures in the absence of substrate.

Similar incubations were conducted at 4° to investigate the relationship between the purported dichlorocarbene–Fe-II-cytochrome P-450 complex and carbon monoxide production. In these incubations the reaction was initiated by the addition of sodium dithionite in Tris buffer (to a final concentration of 1 mM) and the formation of the carbene complex at 460 nm monitored for various time intervals from 1 to 25 min in an Aminco DW2 spectrophotometer in the split beam mode. The anaerobic incubation mixture was oxidized with potassium ferricyanide in Tris buffer (to a final concentration of 2 mM) and allowed to react for 4 min before re-reduction with excess solid sodium dithionite (2–3 mg). Due to a change in the baseline the carbon monoxide production was determined from the ΔE [419 nm (peak) – 409 nm (trough)]. The reference cuvette contained all components except substrate and was treated in an identical manner.

Incubations in deuterated water. The washed rat liver microsomal pellet from the 105,000 *g* centrifugation was resuspended in 0.1 M phosphate buffer, pH 7.6, prepared in ^2H_2O (99.7 %, EGA, Steinheim, F.R.G.). The suspension was centrifuged (105,000 *g*) and the pellet resuspended in the same ^2H_2O-phosphate buffer at 2.23 mg microsomal protein/ml (1.8 nmole P-450/mg protein). Anaerobic incubations (repeated evacuation and gassing with dinitrogen) were performed in 25-ml flasks and contained microsomal suspension (10 ml) and NADPH (1 mM). The reaction was started by the injection of 1 μl CCl_4 (1 mM final concentration) through the rubber stopper and the incubation was continued for 10 min at 37°. After cooling in ice, pentane (3 ml) was introduced to extract the chloroform produced and the separated extract was dried (molecular sieve 3 Å, Merck).

Aliquots of the extract (4 μl) were injected into a gas chromatograph (Varian 1400) linked to a mass spectrometer (Varian MAT 311) to separate the chloroform from the solvent and other extracted lipid soluble materials. The relative abundance of the ions m/e 86.9026 ($^{12}C^1H^{37}Cl_2^+$) and 87.9088 ($^{12}C^2H^{37}Cl_2^+$) was used to determine the relative amounts of C^1HCl_3 and C^2HCl_3 formed in the incubation.

Production of carbon monoxide from iron(II)-protoporphyrin IX dichlorocarbene. Fe(II)-protoporphyrin IX dichlorocarbene (10 mg), prepared according to the method of Mansuy *et al.* [19], was dissolved in methanol (1 ml) under dinitrogen in a closed reaction vessel. Dioxygen-free 0.1 M Tris–HCl buffer, pH 7.6 (5 ml), containing potassium ferricyanide (50 mg) was introduced and the gaseous products displaced with dinitrogen over a period of 1 hr into a cuvette containing heamoglobin (4 μM) in 0.1 M Tris–HCl buffer, pH 7.6. At intervals the dinitrogen flow was stopped and the difference spectrum recorded from 350 to 500 nm against a reference cuvette containing haemoglobin in buffer which had been gassed similarly with dinitrogen.

RESULTS

The results of the large scale incubations of anaerobic reduced microsomal fractions with ^{14}C-labelled carbon tetrachloride are summarized in Fig. 1. In the absence of the microsomal fraction only trace

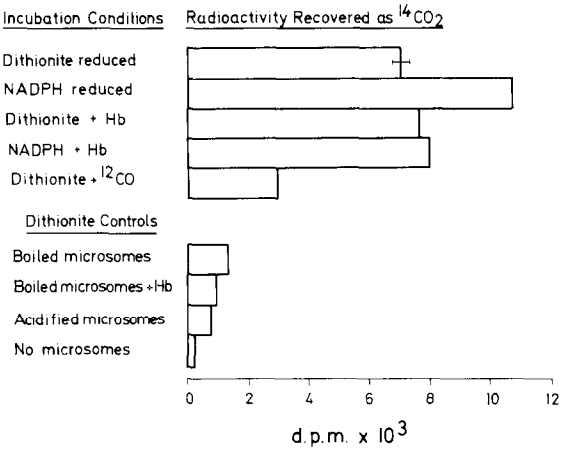

Fig. 1. Formation of ^{14}CO from $^{14}CCl_4$ by anaerobic microsomal suspensions. Incubation in 30 ml 0.1 M Tris–HCl buffer, pH 7.6, contained microsomal protein (3 mg/ml), sodium dithionite (25 mg) or NADPH (1 mM) and 0.9 μCi $^{14}CCl_4$ (1 mM) under anaerobic conditions. Some experiments also contained haemoglobin (Hb) (4 μM). At the end of the 1-hr incubation (30°), carrier ^{12}CO (3 μmoles) and conc. H_2SO_4 (5 ml) were added after cooling to 0°. ^{14}CO was determined after catalytic oxidation to $^{14}CO_2$ by scintillation counting following displacement of the volatile products from the incubation mixture through the apparatus described in Materials and Methods.

amounts of radioactivity were absorbed in the phenylethylamine trap and represented approximately 0.01 per cent of the radioactivity present in the incubations. This radioactivity may represent trace impurities in the $^{14}CCl_4$ used, or small amounts of $^{14}CHCl_3$, formed by a chemical reaction with sodium dithionite, as the silica gel trap was slightly less efficient in retaining $CHCl_3$ than CCl_4 over the 5-hr period of flushing with dinitrogen. The heterogeneous chemical reaction between CCl_4 and dithionite is enhanced by the presence of denatured microsomal fraction and this probably accounts for the greater amounts of radioactivity recovered in the phenylethylamine in these control experiments.

In the presence of sodium dithionite the microsomal fraction produced ^{14}CO (7140 d.p.m.) from $^{14}CCl_4$ without correction for contamination from the non-enzymic reaction. After correction for the non-enzymic reaction, the average yield of ^{14}CO from the 1-hr incubations was 0.99 nmole/mg protein (0.58 nmole/nmole P-450). Attempts to increase the yield of ^{14}CO by scavenging the ^{14}CO produced in the reaction with haemoglobin produced little or no increase. Increasing the amount of carrier ^{12}CO, added after the reaction, from 3 to 30 μmoles did not increase the amount of radioactivity retained by the phenylethylamine trap indicating that all of the ^{14}CO generated in the reaction had been recovered from the reaction mixture and the remainder of the trapping system. Replacement of sodium dithionite by NADPH, in the presence or absence of haemoglobin, gave essentially similar results although in this case the potential contamination of the contents of the phenylethylamine trap is greater because of the comparatively large amounts of $^{14}CHCl_3$ known to be produced under these conditions. The inclusion of saturating amounts of ^{12}CO at the start of the dithionite-supported reaction produced a marked reduction in the yield of ^{14}CO in the 1-hr incubation to 0.29 nmole/mg protein after correction for the non-enzymic reaction (71 per cent inhibition).

Investigation of the aqueous phase following the anaerobic incubation of $^{14}CCl_4$ with microsomal fraction and NADPH showed that the soluble activity, which could be formate, is at most 10 per cent of the CO produced.

The CO production from CCl_4 in dithionite-reduced liver microsomal preparations, measured spectroscopically as carboxyhaemoglobin, yielded a maximum of 5 nmoles/mg protein in a 40-min period and was not linear with time. For a 10-min incubation the yield of CO was between 2.0 and 3.0 nmoles/mg protein (1–1.5 nmoles CO/nmole cytochrome P-450) as an average from different microsomal preparations and was unaffected by the inclusion of glutathione (1 mM). Increasing the substrate concentration to 4 mM decreased the yield of carbon monoxide. The addition of metyrapone inhibited the reaction but high concentrations were required; 0.04 mM metyrapone produced 12 per cent inhibition and 0.4 mM a 70 per cent inhibition of the CO yield in a 10 min incubation. Removal of dioxygen from the system by repeated evacuation and flushing with dinitrogen and replacement of sodium dithionite by NADPH produced essentially similar results. Treatment of the microsomal suspension with sodium cholate, sufficient to convert almost all of the cytochrome P-450 to the P-420 form, resulted in complete inhibition. Carbon monoxide added at the end of the incubation period demonstrated that cholate did not impair the detection and measurement of any CO produced.

Incubation of CCl_4 with dithionite-reduced microsomal fraction at 4° (Fig. 2) in the presence of haemoglobin showed a slow formation of the reduced ligand spectrum with the peak at 460 nm reaching a maximum value in about 20 min. During this time there was no CO production as indicated by the absence of the carboxyhaemoglobin peak at 419 nm in these scans. Subsequent oxidation of the system under anaerobic conditions with ferricyanide and re-reduction with dithionite showed the immediate formation of CO accompanied by a decrease in the magnitude of the absorbance at 460 nm. The latter again increased with time as the original reaction proceeded but there was no further change in the carboxyhaemoglobin peak, unless the system was oxidized again.

When this experiment was repeated for various reaction times a linear relationship was observed between the magnitude of the reduced ligand spectrum (ΔE 460–530 nm) and the amount of CO produced upon oxidation with ferricyanide (ΔE 419–409 nm). The correlation coefficient was $r = 0.94$. The formation of the 460 nm peak was inhibited by inhibitors of cytochrome P-450 like metyrapone and abolished by pretreatment of the microsomal fraction with sodium cholate, which converts the cytochrome P-450 to the P-420 form.

In an incubation of dithionite-reduced microsomal fraction and CCl_4 at 4° in the absence of haemoglobin

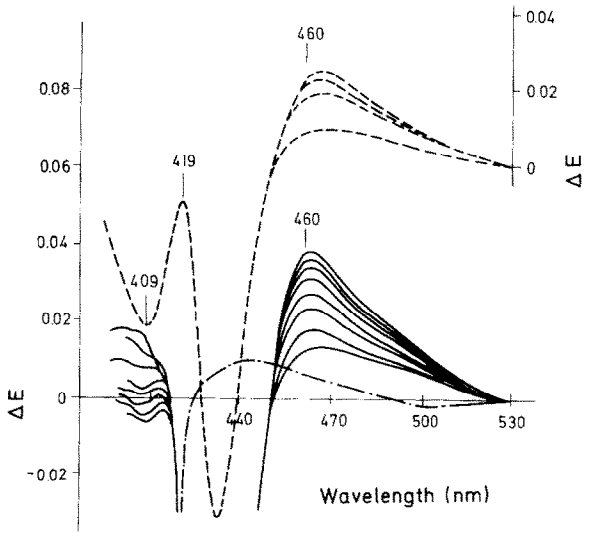

Fig. 2. Production of CO from the reduced cytochrome P-450–CCl_4 ligand complex after oxidation with ferricyanide. The two cuvettes contained 2.5 mg microsomal protein (2.8 nmoles cytochrome P-450/mg) and haemoglobin (4 μM) in 2.5 ml 0.1 M Tris–HCl buffer, pH 7.6 at 4° with CCl_4 (1 mM) in the test cuvette. Both cuvettes were reduced with sodium dithionite in buffer (1 mM, final concentration) and scans taken at 2, 4, 6, 8, 10, 12, 14 and 16 min (——). After this 16 min period both cuvettes were oxidized with potassium ferricyanide solution in buffer (2 mM, final concentration) for 4 min (—·—), re-reduced with solid sodium dithionite and scans taken at 0.3, 1, 2 and 4 min (– – –). The final scans have been displaced vertically by 0.06 extinction units for clarity.

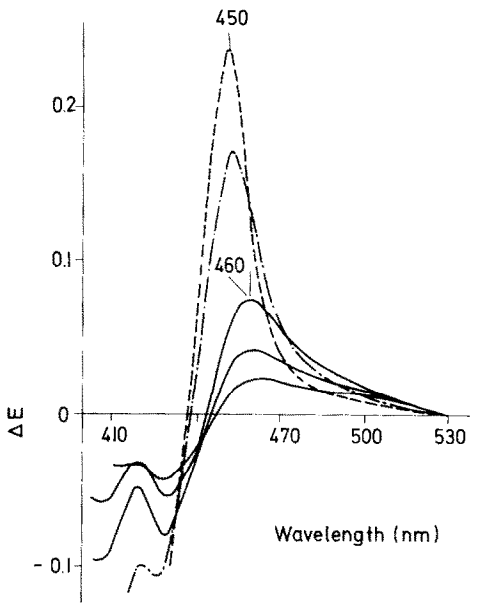

Fig. 3. Reduced cytochrome P-450–CO difference spectra before and after oxidation of the reduced cytochrome P-450–CCl_4 ligand complex with ferricyanide. Incubation conditions were as for Fig. 3 except that microsomal protein contained 3.7 nmoles cytochrome P-450/mg and haemoglobin was omitted. (———) Addition of sodium dithionite solution, scans at 2, 8 and 16 min; (—·—) incubation saturated with CO; (– – –) incubation oxidized with potassium ferricyanide solution for 4 min, re-reduced with solid sodium dithionite and saturated with CO.

(Fig. 3) the peak of the reduced ligand spectrum at 460 nm shifted to 454 nm upon the addition of carbon monoxide due to a mixture of cytochrome P-450 Fe^{2+}–ligand and cytochrome P-450 Fe^{2+}-CO. Oxidation of this mixture with ferricyanide and reduction produced a larger peak now at 450 nm representing the total cytochrome P-450 remaining in the incubation mixture. From such data, for different initial reaction times, it was possible to calculate that after 8 min reaction with CCl_4, 36 per

Table 1. Formation of chloroform from carbon tetrachloride by anaerobic NADPH-reduced microsomal fraction in an environment of ^2H_2O

Incubation conditions	Chloroform produced		
	C^1HCl_3 (% of total)	C^2HCl_3 (% of total)	Total (nmoles/mg protein/10 min)
Microsomes, ^2H_2O, NADPH	92.4	7.6	3.6
	89.6	10.4	4.5
	93.4	6.6	6.3†
Microsomes, ^1H_2O, NADPH	>99.5	<0.5	8.3
Controls			
Boiled microsomes, ^2H_2O, NADPH	99.0	1.0	-
	99.3	0.7	-†
Microsomes, ^2H_2O, no NADPH	98.1	1.9	-

* Incubations under dinitrogen contained 22.3 mg microsomal protein (1.8 nmoles cytochrome P-450/mg), NADPH (1 mM) and CCl_4 (1 mM) in 10 ml 0.1 M KH_2PO_4/K_2HPO_4 buffer, pH 7.6, prepared in ^2H_2O. After incubation at 37° for 10 min, the chloroform produced was extracted with pentane, separated and determined by g.l.c. The ratio of C^1HCl_3 to C^2HCl_3 was determined by high resolution mass spectrometry. Control experiments contained added C^1HCl_3 (270 nmoles).

† Drying of the pentane extract with a molecular sieve was excluded.

Table 2. Comparison of the yield of products from the anaerobic metabolism of carbon tetrachloride and the consumption of NADPH in microsomal incubations*

	Amount (nmoles/mg protein/10 min)	NADPH consumption (nmoles/mg protein/10 min)	
		Calculated	Measured
Chloroform			
total	15.0 ± 1.7		
derived from $\cdot CCl_3$ radical	13.8 ± 2.0†	6.9 ± 1.0	
derived from $^-CCl_3$ carbanion	1.2 ± 0.2	1.2 ± 0.2	
Covalent binding to proteins	2.4 ± 0.2	1.2 ± 0.1	
Covalent binding to lipids	11.0 ± 0.5	5.5 ± 0.3	
Carbon monoxide	2.4 ± 0.4	2.4 ± 0.4	
		17.2 ± 2.0	19.8 ± 1.5

* Incubations under dinitrogen contained 11.3 mg microsomal protein (1.7 nmoles cytochrome P-450/mg), a NADPH regenerating system consisting of glucose-6-phosphate (16 mM), $MgCl_2$ (12 mM), $NADP^+$ (0.5 mM) and glucose-6-phosphate dehydrogenase (1 U/ml), and CCl_4 (1 mM) in 2.2 ml of Tris–HCl buffer, pH 7.6. After incubation at 37° for 10 min chloroform was extracted with pentane (3 ml) and analysed by g.l.c. In similar incubations the covalent binding was determined according Uehleke *et al.* CO was determined as described in Materials and Methods but at 37°. NADPH uptake was measured in identical incubations by monitoring the absorption difference between 366 and 500 nm.

† Calculated according to the results shown in Table 1.

cent of the residual cytochrome P-450 in the cuvette was present as the reduced, ligand complex; at 16 min it was 58 per cent.

The final determination of the total remaining cytochrome P-450 (2.6 nmoles/ml) indicated that approximately 30 per cent of the original cytochrome P-450 (3.7 nmoles/ml) had been destroyed over a 16-min incubation period.

The ferricyanide oxidation of the model carbene complex, Fe(II)-protoporphyrin IX dichlorocarbene, produced CO as indicated by the formation of the typical haemoglobin–CO difference spectrum with a peak at 419 nm (results not shown). This agrees with results by D. Mansuy (personal communication).

In order to compare the extent of the CO production with that of the other processes known to occur with CCl_4 in anaerobic NADPH-reduced microsomal preparations the chloroform production was measured both in a normal microsomal fraction and in a microsomal fraction suspended in a buffer containing ^2H_2O. A long preincubation of 1 hr with ^2H_2O ensured a sufficient equilibration.

Anaerobic incubation of CCl_4 with NADPH-reduced microsomal fraction in an environment of deuterated water gave a lower total yield of chloroform than in normal protonated water (Table 1).

The chloroform produced was mostly in the protonated form with the deuterium label only being incorporated to about 10 per cent in the product. Exclusion of the molecular sieve used to dry the final extract did not affect this value although its use may have given a lower overall recovery.

Chloroform production and the covalent binding of $^{14}CCl_4$ to microsomal lipids and proteins were measured in the same preparation under identical conditions (Table 2).

From this data the presumptive NADPH consumption for the various processes was calculated by assuming that the $\cdot CCl_3$ radical probably responsible for the covalent binding as well as the C^1HCl_3 formation required 0.5 equivalents of NADPH whereas the CCl_3^- carbanion and the carbene responsible for C^2HCl_3 and CO production, respectively, need 1 mole NADPH per mole product. The calculated value almost matches the experimentally determined NADPH oxidation, as measured by the decrease of the absorption difference between 366 and 500 nm.

DISCUSSION

The one-electron reduction of CCl_4 by reduced liver microsomal fractions under anaerobic conditions to form the trichloromethyl radical, $CCl_3\cdot$, had been recognised for some time [1]. The results above show that this radical may either be released from its complex with the ferric form of cytochrome P-450 or, alternatively, undergo further reduction following the supply of reducing equivalents to the cytochrome. This facility for two successive one-electron reductions by cytochrome P-450 is to be expected considering the two electron donor system of the microsomal monooxygenase. A scheme for these reactions involving CCl_4 is proposed in Fig. 4 and shows a partial analogy to the commonly accepted mechanism of dioxygen activation by cytochrome P-450-dependent monooxygenases [12].

The first-formed radical species in this cycle, $Fe^{3+}\cdots CCl_3\cdot$, is formally analogous to the oxy-complex ($Fe^{3+}O_2^-$) involved in oxidative metabolism. The former complex is likely to be rather unstable and to release the $CCl_3\cdot$ radical, a process that is equivalent to the loss of a superoxide radical anion

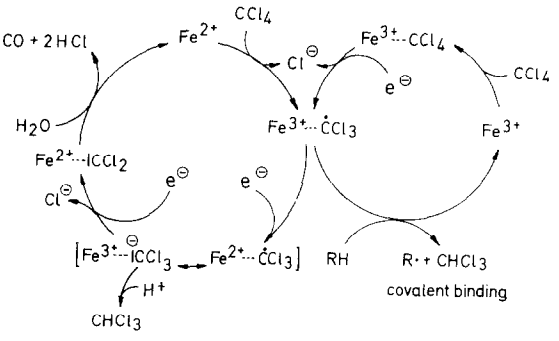

Fig. 4. Proposed mechanism for the formation of carbon monoxide from CCl_4 by cytochrome P-450 under anaerobic conditions. Fe^{2+} and Fe^{3+} represent the haem binding site of cytochrome P-450

(O_2^-) from the dioxygen analogue [20]. Once released from the complex, the $CCl_3\cdot$ radical will abstract a hydrogen atom from its immediate environment to form chloroform. Thus, in anaerobic NADPH-reduced incubations of CCl_4 in deuterated water, the major part of the chloroform produced contains hydrogen, derived from the bio-material, rather than deuterium from the reaction solvent (Table 1). Indeed, the membrane or cytochrome P-450 itself may suffer damage and denaturation by this process.

As an alternative to its homolytic breakdown, the Fe^{3+}···$CCl_3\cdot$ complex can accept an electron to form Fe^{2+}···$CCl_3\cdot$, which in its mesomeric structure would be analogous to the peroxo complex Fe^{3+}···O_2^{2-}. Both species are probably not stable but in the presence of a proton may yield chloroform or the active oxygen complex $[FeO]^{3+}$ [12], respectively. The small amount of deuterated chloroform produced during the incubations with ^2H_2O cannot be accounted for by a simple exchange reaction between $HCCl_3$ and ^2H_2O but may well originate from the carbanion complex with water protons. The low yield of chloroform in ^2H_2O probably is due to an isotope effect as seen in microsomal monooxygenations performed in ^2H_2O [21].

From our data it is very likely that release of a chloride ion from the carbanion complex occurs after reduction to the ferrous state to form a cytochrome P-450–Fe^{2+} dichlorocarbene complex, to which we attribute the spectrum at 460 nm. Such a formation of carbenes from trihalocarbanions has ample precedent in organic chemistry [22] and a very close model exists in the reduction of CCl_4 by Fe(II) tetraphenylporphyrin complexes to the corresponding dichlorocarbene complex [23]. Since this model also gave carbon monoxide after oxidation, our mechanism of CO formation from the cytochrome P-450 dichlorocarbene is chemically well-documented. It could even be shown that the decrease in absorbance at 460 nm correlates with the amount of CO evolved. The free $CCl_3\cdot$ radical also cannot be responsible for CO formation because glutathion stimulates chloroform formation but fails to stimulate CO production. If a free carbanion would be a precursor of CO then more deuterated C^2HCl_3 should have been detected in the isotope experiment because protonation would have been much faster than hydrolysis [22].

After the liberation of the dichlorocarbene by ferricyanide oxidation the cytochrome P-450 forms back its CO complex upon reduction (Fig. 3). This indicates that the enzyme seems to emerge undamaged from the cycle. Due to the stability of the carbene complex under anaerobic conditions its cycle of formation (Fig. 4, left panel) is slower than the cycle involving the radical (Fig. 4, right panel). This agrees with the overall electron balance sheet which indicates that most of the products are derived from the $CCl_3\cdot$ radical and only about 15 per cent for the hydrolysis of the carbene complex.

The mechanism proposed for the reduction of CCl_4 demonstrates the potential of the microsomal monooxygenase to perform reductive reactions and is useful as a model for several other reductive dehalogenation reactions of polyhalogenated alkanes at haem centres.

It is difficult to predict what changes in the mechanism occur by introduction of dioxygen into the system. Certainly reductive metabolism of CCl_4 continues and the radical is formed especially when the dioxygen pressure is as low as under *in vivo* conditions in the liver cell. It is well known that the $CCl_3\cdot$ radical can set off lipid peroxidation, but it was also shown previously that the dichlorocarbene complex appears under a limited dioxygen pressure [11]. According to Mansuy [23] this complex could undergo cleavage by molecular oxygen and form phosgene, which will react with cell constituents. This additional aspect of CCl_4-toxicity warrants further investigation.

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