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THE MECHANISM OF THE SUICIDAL, REDUCTIVE INACTIVATION OF MICROSOMAL CYTOCHROME P-450 BY CARBON TETRACHLORIDE

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Abstract—1. Stoichiometric losses of microsomal haem and cytochrome P-450 were observed when carbon tetrachloride (CCl_4) was incubated anaerobically with rat liver microsomes using NADPH or sodium dithionite as a reducing agent. A rapid destruction of haem was also observed during the non-enzymatic reductive incubation of CCl_4 with soluble haem preparations (methaemalbumin) in presence of sodium dithionite. The results indicate that haem is both the site and the target of the suicidal activation of CCl_4 by cytochrome P-450.

2. When an additional, fluorimetric assay for haem determination was used, an equimolar loss of protoporphyrin IX fluorescence was also observed in both the enzymatic and non-enzymatic system, indicating that the haem moiety of cytochrome P-450 has undergone a structural change, involving either loss or labilization of the porphyrin tetrapyrrolic structure. In both systems the loss of porphyrin was prevented by carbon monoxide (CO).

3. A dichlorocarbene–cytochrome P-450 ligand complex is partially responsible for the difference spectrum obtained on addition of CCl_4 to anaerobically reduced rat liver microsomes. A molar extinction coefficient for this complex has been calculated. The carbene trapping agent 2,3-dimethyl-2-butene (DMB) strongly inhibited (>95%) the formation of this spectrum but did not modify the loss of haem in reduced CCl_4-supplemented microsomal incubations. The results suggest that dichlorocarbene ($:CCl_2$) is not significantly involved in CCl_4-dependent haem destruction.

4. Pretreatment of rats with different microsomal enzyme inducers was responsible for similar but not identical patterns of $:CCl_2$ and CO formation and haem loss during incubation of CCl_4 with reduced microsomes. This indicates a critical role of CCl_4 metabolism in the suicidal destruction of cytochrome P-450 haem and suggests that the apoprotein of cytochrome P-450 is capable of modulating not only the metabolism of CCl_4 to $:CCl_2$ but also the hydrolysis of $:CCl_2$ to CO.

5. Inactivation of cytochrome P-450 by CCl_4 with reduced microsomes from Aroclor-pretreated rats was saturable and followed pseudo first-order kinetics. This provides further evidence to conclude that CCl_4 activation is a suicidal process where the reactive metabolite(s) formed bind to haem, we predict, in a one to one stoichiometry.

6. The partition ratio between loss of cytochrome P-450 haem and CCl_4 metabolism by liver microsomes from Aroclor pretreated rats has been investigated using limiting concentrations of CCl_4. It was calculated that approximately 26 molecules of CCl_4 had to be metabolised to achieve the loss of one molecule of haem.

During anaerobic metabolism of carbon tetrachloride (CCl_4) by NADPH-reduced liver microsomes [1, 2] cytochrome P-450 is rapidly inactivated [3, 4] and protohaem, the prosthetic group of cytochrome P-450, is lost [5, 6]. It has been proposed that reactive metabolites such as the trichloromethyl radical ($\cdot CCl_3$) formed during the reductive microsomal activation of CCl_4 [7, 8] and capable of binding covalently to microsomal lipid and protein both *in vitro* [9–11] and *in vivo* [12, 13], may attack and irreversibly modify the prosthetic group [14, 15] or the apoprotein [16] of cytochrome P-450. Consistent with this hypothesis is the observation that the forms of cytochrome P-450 more likely to activate CCl_4 to $\cdot CCl_3$, such as those induced by pretreatment of rats with phenobarbitone (PB) or ethanol, are also more susceptible to CCl_4-dependent destruction [17, 18].

However, direct evidence as to which reactive metabolite is responsible for haem and cytochrome P-450 destruction is still missing. CCl_4 may undergo one or two subsequent one electron reductions to form $\cdot CCl_3$ and dichlorocarbene ($:CCl_2$) respectively. The latter species has been also identified as a reactive metabolite of CCl_4 [19] and could, therefore, conceivably attack the haem moiety and/or the apoprotein of cytochrome P-450 leading to the destruction of the haemoprotein [6].

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‡ Abbreviations used: ALA, aminolaevulinic acid; DMB, 2,3-dimethyl-2-butene; β-NF, β-naphthoflavone; PB, phenobarbitone; Hb, haemoglobin; MHA, methaemalbumin; CO–P-450, CO–cytochrome P-450 complex; CO–Hb, CO–haemoglobin complex; $:CCl_2$–P-450, $:CCl_2$–cytochrome P-450 complex; TBA, tetrabutylammonium hydroxide.

In the present study we have investigated the molecular mechanism of the anaerobic CCl_4-dependent destruction of microsomal cytochrome P-450. In particular, the hypothesis that protohaem is the primary, suicidal target of the metabolic activation of CCl_4 by cytochrome P-450 has been tested. We have also made use of a selective trapping agent to obtain spectral evidence for the formation of $:CCl_2$ during the reductive metabolism of CCl_4 by liver microsomes and to study the role played by this species in the process of haem destruction. Finally, the effect of the pretreatment of rats with different inducers of cytochrome P-450 isoenzymes on both the haem loss and the formation and stability of this metabolite has been investigated.

Some preliminary results of this work have been reported [20, 21].

MATERIALS AND METHODS

Chemicals. $NADP^+$, catalase (1.11.1.6), glucose oxidase (1.1.3.4), glucose-6-phosphate dehydrogenase (1.1.1.49), human albumin, protoporphyrin IX dimethyl ester, hemin and glucose-6-phosphate were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). NADPH was purchased from P-L Biochemicals Inc. (Milwaukee, WI) and carbon monoxide (CO) from BOC (London, U.K.). Aroclor 1254 was a gift of Dr. C. Ioannides, University of Surrey, Guildford. 2,3-Dimethyl-2-butene (DMB) and β-naphthoflavone (β-NF) were purchased from Aldrich Chemical Co. Ltd. (Gillingham, Dorset, U.K.) and phenobarbitone (PB) from BDH Chemicals Ltd. (Poole, Dorset, U.K.). [5-^{14}C]-amino laevulinic acid ([^{14}C]-ALA) was purchased from New England Nuclear Research Products (Boston, MA).

[^{14}C]-Haem (97 nCi/μmole) was prepared biosynthetically from [^{14}C]-ALA using a chicken haemolysate incubation, as described by Dresel and Falk [22] and was then obtained and recrystallized (>95% pure) as described by Labbe and Nishida [23]. Methaemalbumin (MHA), a water soluble complex of human albumin with haem, was prepared by the method of Tenhunen *et al.* [24], using either unlabelled or ^{14}C-labelled haem. Haemoglobin (Hb) was prepared from rat blood by the method of Rossi-Fanelli and Antonini [25].

All other chemicals were obtained from commercial sources and were of analytical grade.

Treatment of animals. Male Wistar Albino rats (150–200 g) bred in the University of Surrey Animal Unit were given Spratt's Laboratory Animal Diet I and water *ad lib.* When indicated, animals were pretreated with inducers of cytochrome P-450 isoenzymes by intraperitoneal injections of PB (80 mg/kg/day for three days) dissolved in 0.9% (w/v) NaCl, Aroclor 1254 (one single injection of 500 mg/kg) or β-NF (80 mg/kg/day for three days) dissolved in corn oil. Control rats were injected with the corresponding volumes of corn oil (approx. 0.5 ml/day for three days).

Preparation of microsomes. Rats were starved for 18 hr before being killed by decapitation 24 hr after the last injection of corn oil, PB or β-NF, and four days after the single injection of Aroclor. Livers were perfused *in situ* through the inferior *vena cava* with approx. 50 ml of ice-cold 0.9% (w/v) NaCl and homogenised in four volumes of 0.25 M sucrose in a glass Potter homogeniser. The homogenates were centrifuged in a MSE-HS 18 centrifuge at 9000 *g* and 4° for 20 min to obtain the post-mitochondrial supernatant. This was centrifuged in a Beckman L5-65 ultracentrifuge at 114,000 *g* and 4° for 1 hr. The microsomal fraction was washed once with 1.15% (w/v) KCl, and finally suspended in 0.1 M Na_2HPO_4 buffer, pH 7.4, containing 20% glycerol, flushed with oxygen-free nitrogen (N_2) and stored at −80°.

Incubations and oxygen-scavenging system. All incubations were performed in rubber stoppered 5 ml glass tubes or 3 ml, 1 cm light path spectrophotometer cells, in the presence of an oxygen-scavenging system to insure anaerobic conditions and to prevent lipid peroxidation. The following additions were routinely made (final concentration in parentheses): catalase (600 U/ml), glucose oxidase (12.5 U/ml) and D-glucose (60 mM). Care was taken to remove oxygen from the buffer and from the incubation vessel by flushing O_2-free N_2 through two needles inserted into suitable rubber stoppers as described by Cooper *et al.* [26]. Under these conditions the oxygen concentration in the incubations was consistently below the lower limit of detection by the oxygen electrode. After a 5 min preincubation, subsequent additions were made using, whenever possible, solutions saturated with N_2. The incubation mixture, unless otherwise indicated, contained in 0.1 M Na_2HPO_4 buffer, pH 7.4, the following components with final concentration in parentheses: microsomal protein (1 mg/ml), NADPH (1 mM) or sodium dithionite (2 mg/ml), CCl_4 (1 mM) and, where appropriate, DMB (5 mM). The last two reagents were added in methanol. In some experiments MHA containing unlabelled or ^{14}C-labelled haem was similarly incubated under anaerobic conditions with CCl_4 and/or sodium dithionite. In other experiments the following NADPH regenerating system was used (final concentration in parentheses): glucose-6-phosphate dehydrogenase (0.15 U/ml), glucose-6-phosphate (1.9 mM) and $NADP^+$ (0.12 mM).

Determination of cytochrome P-450, haem and protoporphyrin IX. Cytochrome P-450 was measured in oxidised stock microsomes by the method of Omura and Sato [27] using a dual beam UV-VIS spectrophotometer mod. Varian 2200, and in reduced anaerobic incubations by the method previously described [28] using a dual wavelength spectrophotometer mod. Perkin Elmer 356. Microsomal haem was assayed by the pyridine/haemochrome method [29] using either the reduced absolute spectrum or the reduced minus oxidised difference spectrum and a εmM (557–541) = 20.7 [30] for both types of spectra. Microsomal haem was also measured by conversion to protoporphyrin IX by the oxalic acid method [31] and determination of the fluorescence at 404/603 nm (excitation/emission wavelengths, respectively) using a Perkin Elmer LS-5 spectrofluorimeter. Solutions of authentic protoporphyrin IX of known concentration were used as the standard. Microsomal protein was assayed by

the method of Lowry *et al.* [32], using bovine serum albumin as the standard.

Determination of CO *and* $:CCl_2$. The amounts of CO and $:CCl_2$ formed from CCl_4 were concurrently measured in the same NADPH-reduced incubation by the difference spectrum obtained in the presence of 3 μM Hb [33] between a sample cuvette (to which CCl_4 had been added) and a corresponding reference cuvette which contained no CCl_4. The CO present in the incubation mixture was quantified from the difference in absorbance between the peak at 419 nm of CO–Hb and its isosbestic point at 411 nm, using a calibration curve constructed by serial additions of known amounts of CO to a mixture containing both Hb and microsomes from PB-pretreated rats in presence of NADPH. The broad peak at 460 nm, which is thought to be due to a $:CCl_2$–cytochrome P-450 complex ($:CCl_2$–P-450), was employed to quantitate the carbene species (once the contaminating CO had been displaced from cytochrome P-450 and trapped onto Hb) using a ε mM (460–530) = 56.2. This was obtained, as described in more detail in the Results section, by calculating the portion of total cytochrome P-450 complexed to carbene and relating it to the difference in absorbance between 460 and 530 nm, assuming a 1:1 carbene/cytochrome P-450 ratio in the complex.

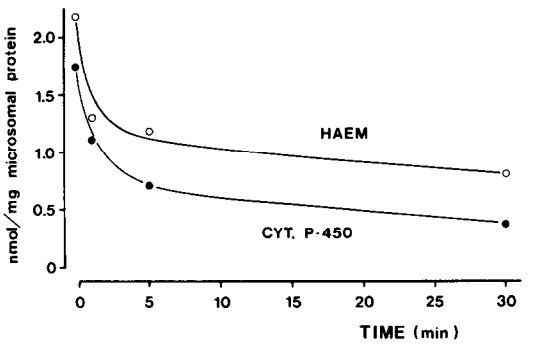

Fig. 1. Equimolar loss of cytochrome P-450 and haem during the anaerobic incubation of NADPH-reduced rat liver microsomes with CCl_4. Incubations were at 37° under oxygen-free N_2 in rubber-stoppered glass cuvettes and contained (final concentration in parentheses) liver microsomal protein (0.74 mg/ml), the NADPH regenerating system described under Materials and Methods and CCl_4 (1.07 mM) in 2.5 ml 0.1 M Na_2HPO_4 buffer, pH 7.4, in presence of the oxygen-scavenging system described under Materials and Methods. The reaction was terminated either by cooling to 0° and immediately injecting into the incubation mixture 100 μl of a CO-saturated solution of sodium dithionite (50 mg/ml of 0.1 M Na_2HPO_4 buffer, pH 7.4) for cytochrome P-450 determination, or by pipetting 0.9 ml of incubation mixture into 2.1 ml pyridine–NaOH for haem assay. When either CCl_4 or NADPH was omitted the loss of both cytochrome P-450 and haem was negligible over a period of 30 min. Values are means from 2 experiments.

RESULTS

"Suicidal" destruction of cytochrome P-450 by CCl_4 *involves parallel destruction of haem prosthetic group*

The loss of cytochrome P-450 occurring anaerobically on addition of CCl_4 to NADPH-reduced liver microsomes from PB-pretreated rats was compared with the loss of haem measured in the same conditions (Fig. 1). A 60% loss of cytochrome P-450 was observed over a period of 5 min. The loss of haem, as measured by the pyridine/haemochrome method, was parallel and equimolar to that of cytochrome P-450 when monitored over a period of up to 30 min. When sodium dithionite was used as a reducing agent instead of NADPH, cytochrome P-450 was also lost, to a similar extent, on addition of CCl_4 to anaerobic microsomal incubations and here again the loss of cytochrome was accompanied by an equimolar loss of microsomal haem (data not shown). In order to exclude that the loss of the pyridine-haemochrome reaction might be due to masking of the haem iron, making it inaccessible to the pyridine, the haem was also measured by conversion to protoporphyrin IX and determination of the resulting fluorescence [31]. Equimolar losses of the pyridine haemochrome and protoporphyrin IX were observed (Table 1), suggesting that on CCl_4 treatment protohaem undergoes a structural change in its tetrapyrrolic macrocycle. The fluorimetric technique also allowed us to test whether the CCl_4-dependent haem loss could be inhibited by saturating concentrations of exogenous CO. This effect could not be studied [6] using the pyridine/haemochrome method as CO apparently prevented the interaction of pyridine with the reduced haem iron (data not shown). A strong protection by CO against the CCl_4-dependent loss of haem was found (Table 1).

Non-enzymatic "suicidal" activation of CCl_4 *by haem*

Since haem itself is readily reduced by sodium dithionite, we investigated the possibility that CCl_4 might be activated in a non-enzymatic system where haem alone plays the role of the "suicidal" activator. A dramatic loss of haem was observed (Table 2) when MHA was incubated anaerobically with CCl_4/dithionite at concentrations similar to those used in the experiments with microsomes. This too was accompanied by equimolar loss of the haem tetrapyrrolic macrocycle, as measured by the porphyrin fluorescence assay (results not given).

When MHA containing [^{14}C]-haem was incubated anaerobically without any addition (control) or in presence of CCl_4/dithionite there was no loss of radioactivity in either case (recovery of radioactivity was 102 and 105%, respectively) suggesting that no radioactive volatile products of haem had been formed. After incubation, the radioactivity was extracted into two volumes of 80% methanol containing 20 mM tetrabutylammonium hydroxide (TBA) buffered in phosphate and the extracts were then injected into the HPLC system involving a C_{18} reversed phase column, as described elsewhere [21]. Recovery of injected radioactivity from control and CCl_4/dithionite incubations was 94 and 93%, respectively. Both the absorbance at 400 nm and the radioactivity associated with the haem peak markedly decreased after incubation with CCl_4/dithionite (Fig. 2). However, whereas the radioactivity lost from the haem peak was almost completely recovered in several new fractions eluted immediately before and after haem, the recovery of the 400 nm absorbance in

Table 1. CCl_4-dependent loss of haem as measured by either the pyridine/haemochrome reaction or the fluorimetric assay of haem-derived protoporphyrin IX

	Microsomal haem (nmol/mg protein) as: Pyridine Haemochrome	Protoporphyrin IX‡
Control	3.45 ± 0.20 (100%)	3.35 ± 0.23 (100%)
+dithionite	3.68 ± 0.20 (106%) NS	3.15 ± 0.30 (94%) NS
+dithionite and CCl_4	2.15 ± 0.16 (62%)†	2.06 ± 0.19 (61%)*
+dithionite, CCl_4 and CO	—	2.96 ± 0.15 (88%) NS

NS non-significant.
* $P < 0.01$.
† $P < 0.001$.
‡ The yield of haem-derived protoporphyrin IX was determined (porphyrin/fluorescence method) by comparison with fluorescence of authentic protoporphyrin IX standards.

Rat liver microsomes were incubated anaerobically for 10 min at 23° as indicated in the legend to Fig. 1, except that 5 ml tubes were used and reduction was by sodium dithionite (2.5 mg/ml). CO was bubbled through the microsomal suspension for 30 sec before addition of dithionite. At the end of the incubation 100 μl portions of each mixture were added to 1.9 ml saturated oxalic acid (porphyrin assay) and 0.7 ml portions to 1.63 ml of pyridine/NaOH (pyridine/haemochrome reaction). Values are means ± SD of 3 determinations, with percentage of corresponding control value given in parentheses.

these new peaks was only negligible. This suggested conversion of haem into products which retained quantitatively the original haem radioactivity but showed considerable less absorbance at 400 nm. It should be noted that in parallel experiments to be described elsewhere, conducted with unlabelled haem and ^{14}C-labelled CCl_4, approximately 20% of the total radioactivity was eluted in the HPLC fractions containing haem-derived products.

Spectral evidence for CO *and* $:CCl_2$ *formation*

When reduced liver microsomes were incubated with CCl_4 under anaerobic conditions a difference spectrum appeared (as compared with appropriate controls not treated with CCl_4) showing an absorption peak at approximately 454 nm, in agreement with previous findings [2, 33–35]. This spectrum is in fact the result of two components. One component, attributed to $:CCl_2$–P-450, shows a peak at 460 nm; the other, due to the complex of CCl_4-derived and endogenously generated CO with reduced cytochrome P-450 haem (CO–P-450), gives the classical 450 nm peak. In the presence of exogenous Hb, CO–P-450 is not demonstrable in the incubation mixture as the CO is trapped instead as CO–Hb, giving a typical, sharp peak at 419 nm which can be used for quantitation (see Materials and Methods).

The following attempt was made to calculate the ε mM (460–530) of the $:CCl_2$–P-450. After incubating PB microsomes with CCl_4 and sodium dithionite for 10 min at 4° in presence (A) or absence (B) of Hb, the following subfractions of cytochrome P-450 were estimated at the end of the incubation: (a) the amount of haemoprotein destroyed, by determining in (B) the loss of haem due to incubation; (b) the cytochrome available for further ligand formation—over that already complexed by either CO or $:CCl_2$—by adding to sample (B) saturating amounts of CO at the end of incubation and determining the increase in CO–P-450 spectrum; (c) the cytochrome complexed with CCl_4-derived endogenous CO, by subtracting from the ΔA (450–490) of the sample incubated without Hb (B) the corresponding ΔA measured in the sample containing Hb (A); and, finally, (d) the portion of cytochrome complexed by carbene, by subtracting from the total cytochrome P-450 remaining after incubation the subfractions (b) and (c) above. Using this method the ε mM (460–530) for $:CCl_2$–P-450 was calculated to be 56.2. It must be emphasized that the above ε mM value can only be considered as approximate and confirmation by a more direct approach would be desirable.

Table 2. CCl_4-dependent loss of haem during anaerobic incubation of methaemalbumin with sodium dithionite

Incubation	HAEM (nmol/ml)
Control	3.76 ± 0.06
+CCl_4	3.69 ± 0.02 NS
+dithionite	3.47 ± 0.06 NS
+CCl_4 and dithionite	0.61 ± 0.06*

NS non-significant.
* $P < 0.001$.

Anaerobic incubations were for 5 min at 20° and contained (final concentration in parentheses) methaemalbumin (4 nmol/ml), sodium dithionite (2.9 mM) and/or CCl_4 (1 mM) in 2.5 ml 0.1 M Na_2HPO_4 buffer, pH 7.4 containing the usual oxygen scavenging system. The reaction was terminated by pipetting 0.8 ml of the mixture into 1.87 ml pyridine/NaOH for haem assay. Values are expressed as mean ± SD of 3 determinations.

Effect of a carbene-trapping agent on CCl_4*-dependent cytochrome P-450 destruction*

In order to investigate whether $:CCl_2$ contributed as a reactive species to the destruction of the haemoprotein, the specific carbene-trapping agent DMB was included in the incubation mixture. DMB caused a strong (>95%) inhibition of the difference spectrum obtained when dithionite- or NADPH-reduced liver microsomes from PB-pretreated rats were incu-

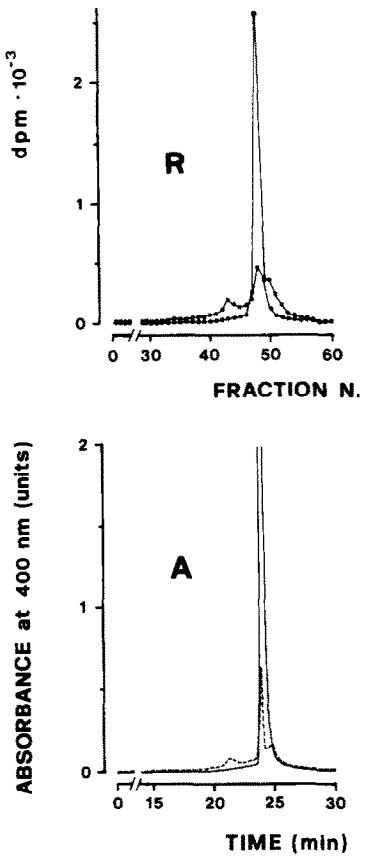

Fig. 2. Absorbance at 400 nm (A) and recovery of radioactivity (R) in the HPLC eluate after anaerobic incubation of [^{14}C]-haem in the absence and presence (——,--- in A and ●—●, ■—■ in R, respectively) of CCl_4/dithionite. Both incubations were for 5 min at 25° in stoppered minivials and contained (final concentration in parentheses) [^{14}C]-haem (1.25 mM, 97 nCi/μmol) in 60 μl of 0.1 M Na_2HPO_4 buffer, pH 7.4 in presence of the usual oxygen scavenging system. At time 0 sodium dithionite and CCl_4 (both 2 mM) were added to one incubation mixture. At the end of the incubation the haem or haem-derived pigments were extracted in methanol/TBA, injected into a reversed phase HPLC system and eluted by gradient mixtures of methanol and water, containing 2.5 to 1 mM TBA. After detection of absorbance in the eluate, radioactivity was collected in 0.5 min fractions and counted.

bated with CCl_4 in the absence of Hb. This provided additional evidence for the formation of a carbene–cytochrome P-450 ligand complex during the reductive anaerobic metabolism of CCl_4 by liver microsomes. However, DMB did not significantly prevent the CCl_4-dependent loss of haem in these dithionite-reduced incubations (25% and 22% in the absence and the presence of DMB, respectively) nor in NADPH-reduced incubations. Values of haem found (averages ± SD of 3 observations) after 10 min incubation of liver microsomes from PB-pretreated rats with appropriate additions were as follows: 2.63 ± 0.04, controls; 2.47 ± 0.03, NADPH; 1.62 ± 0.03, NADPH and CCl_4; 1.69 ± 0.02, NADPH, CCl_4 and DMB. In parallel incubation mixtures also containing 3 μM Hb, the carbene peak at 460 nm was again inhibited by DMB and no significant CO–Hb peak at 419 nm could be observed (data not shown).

Role of CCl_4 metabolism in cytochrome P-450 haem destruction

The effect of several rat pretreatments on CCl_4-dependent reductive destruction of microsomal haem was investigated and related to the production of $:CCl_2$ and CO by the same types of microsomes. The production of the two metabolites was studied at 4° so as to make their formation slower and easier to measure. PB and Aroclor pretreatment markedly stimulated both the formation of the two metabolites (Table 3) and also the extent of haem loss (Table 4). β-NF was on the other hand inactive in both respects when compared with control. The amount of CO produced is dependent on the hydrolysis of $:CCl_2$, which presumably takes place rapidly once the latter species has become dissociated from cytochrome P-450. On this assumption, the ratio of carbene to CO can be considered as an index of the stability of $:CCl_2$–P-450. The increase of this ratio shown by the forms of cytochrome P-450 induced by treatment with PB or Aroclor, but not β-NF or corn oil (Table 3), suggests that the carbene complexes of these induced forms have a greater stability, when compared with the carbene complexes of microsomes from control or β-NF-pretreated rats.

Inactivation kinetics and determination of partition ratio between catalytic activity and "suicidal" inactivation of cytochrome P-450

Preliminary experiments showed that CCl_4-dependent cytochrome P-450 destruction is a saturable time- and dose-dependent process which apparently follows pseudo first-order kinetics. A maximum 60% loss of cytochrome P-450 haem was achieved after 15–20 min incubation of liver microsomal preparations from Aroclor pretreated rats with high concentrations of CCl_4 (1–5 μmol/mg protein) and 1 mM NADPH at 22° (data not shown). In Fig. 3 the log % of the inactivable haemoprotein initially present was plotted against incubation time after addition of 30 μM CCl_4 to the NADPH-supplemented incubation mixture. The loss of cytochrome P-450 occurred in a pseudo first-order biphasic manner with half-times of 3.2 and 28.9 min. Pseudo first-order kinetics have been reported for enzyme inactivation by "suicide" substrates [36, 37].

When concentrations of CCl_4 two orders of magnitude lower than those needed to achieve a maximum loss were used, the haemoprotein was still rapidly inactivated but the final loss was reached earlier and it was significantly smaller, suggesting that limiting amounts of CCl_4 had been used. In one of these experiments the total loss of haem obtained with limiting concentrations of CCl_4 (15 nmol/mg protein) was investigated and the effect of additional NADPH and/or CCl_4 was also studied (Fig. 4). Aroclor microsomes were incubated anaerobically at 22° with CCl_4/NADPH for various periods of time up to 30 min: no further haem loss was observed after 10–15 min of incubation probably due to no more substrate being available for the reaction. This

Table 3. Effect of rat pretreatment on the CCl_4-dependent formation of CO and $:CCl_2$ by NADPH-reduced rat liver microsomes

	Rat pretreatment	Time (min)			
		1	5	10	15
	C	0.035	0.064	0.089	0.107
$:CCl_2$	PB	0.392	0.518	0.589	0.696
	A	0.228	0.428	0.518	0.546
(nmol/mg prot)	β-NF	0.053	0.071	0.107	0.142
	C	0.24	0.49	0.83	0.97
CO	PB	0.74	1.10	1.52	1.85
	A	0.35	0.82	1.43	1.80
(nmol/mg prot)	β-NF	0.29	0.55	0.86	1.15
	C	0.15	0.13	0.11	0.11
$:CCl_2$/CO	PB	0.53	0.47	0.39	0.38
	A	0.65	0.52	0.36	0.30
	β-NF	0.18	0.13	0.12	0.12

Microsomes (1 mg protein) from control rats or rats pretreated with an inducer were incubated anaerobically in 1 ml cuvettes, a test and a reference cuvette being used for each microsomal preparation. The initial cytochrome P-450 concentration was 1.5, 2.1, 2.5 and 1.6 nmol/mg protein in microsomes from control (C), phenobarbitone (PB), Aroclor (A) and β-naphthoflavone (β-NF) pretreated rats, respectively. The incubation mixture contained in 1 ml 0.1 M Na_2HPO_4 buffer, pH 7.4, Hb (3 μM) and the NADPH generating and oxygen scavenging systems. The reaction was started by injecting CCl_4 into the test cuvette (to a 1 mM concentration) and, after incubation at 4° for various periods of time, the difference spectrum between the test and reference cuvettes was recorded between 530 and 390 nm. CO and $:CCl_2$ were measured as their ligand complexes with haemoglobin and cytochrome P-450, respectively. Rat pretreatment regimens are described under Materials and Methods.

The ratio of $:CCl_2$ to CO produced by different microsomal preparations has also been calculated and the steady state value reached at 10–15 min has been used to compare (see text) the stability of the complex in different microsomes. With microsomes from rats pretreated with Aroclor or phenobarbital the stability of $:CCl_2$–P-450 was two to three fold higher than with microsomes from β-naphthoflavone- or corn oil-pretreated animals.

interpretation is based upon the following additional observations: (a) the amount of cytochrome P-450 available for destruction was not limiting as 50–55% of the "suicide-prone" enzyme was known, from preliminary experiments, to be still undamaged; (b) NADPH was also not limiting as a new addition of NADPH at 30 min did not cause any further loss of enzyme; (c) on the other hand, a new larger dose of CCl_4 at 30 min, whether added on its own or together with NADPH, caused a further significant (approx. 40%) loss of inactivable enzyme. It was therefore assumed that all the substrate originally present had been metabolized within 15 min. Based on this assumption the partition ratio [36] between metabolic and suicidal events (i.e. how often during CCl_4 metabolism will the catalytic cycle of cytochrome P-450 result in haem destruction) was calculated. Very similar values of partition ratio were obtained using two different concentrations of substrate (Table 5), suggesting that approximately 26 molecules of CCl_4 had to be metabolized on average for every molecule of cytochrome P-450 inactivated.

Table 4. Effect of rat pretreatment on the CCl_4-dependent loss of cytochrome P-450 haem from NADPH-reduced liver microsomes

Pretreatment	Microsomal haem (nmol/mg protein)		Loss of haem	
	initial	final	(nmol/mg)	(% of initial value)
Corn oil	1.48 ± 0.04	1.01 ± 0.07	0.47	32
PB	2.35 ± 0.10	1.31 ± 0.01	1.34	44
Aroclor	2.92 ± 0.13	1.37 ± 0.07	1.55	53
β-NF	1.76 ± 0.02	1.34 ± 0.04	0.42	24

Anaerobic incubations were for 10 min at 25° and contained (final concentrations in parentheses) 1 mg microsomal protein (0.77, 1.62, 2.40 and 1.17 nmol cytochrome P-450/mg for corn oil, PB, Aroclor and β-NF microsomes, respectively), NADPH (1 mM) and CCl_4 (1 mM) in 1.5 ml 0.1 M Na_2HPO_4 buffer, pH 7.4 in presence of the usual oxygen scavenging system. Values are mean ± SD from at least 3 determinations.

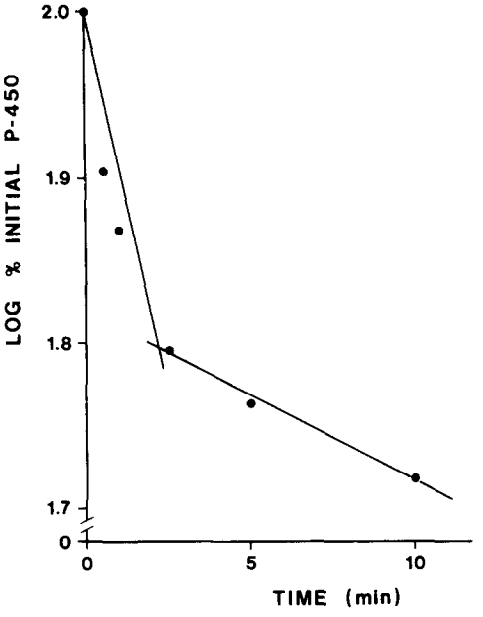

Fig. 3. Inactivation kinetics of microsomal cytochrome P-450 by CCl_4. The % of cytochrome P-450 susceptible to inactivation (1.45 nmol/mg protein, equal to approximately 60% of the total cytochrome P-450) remaining in the incubation mixture was plotted on a log scale against time of incubation after addition of CCl_4. Inactivation was biphasic with calculated half-times of 3.2 and 28.9 min. Anaerobic incubations were at 22° and contained 1 mg microsomal protein (2.4 nmol cytochrome P-450/mg) from the liver of Aroclor-pretreated rats and 1 mM NADPH in 2 ml 0.1 M Na_2HPO_4 buffer, pH 7.4 in presence of the oxygen scavenging system described under Materials and Methods. At time 0 CCl_4 (30 μM final concentration) was added to the incubation mixture. At the indicated times 0.8 ml of the incubation mixture were used for haem assay. Decrease of microsomal haem indicated cytochrome P-450 loss, since under anaerobic conditions cytochrome b_5, the other haemoprotein present in significant amounts in microsomes, is not vulnerable to CCl_4.

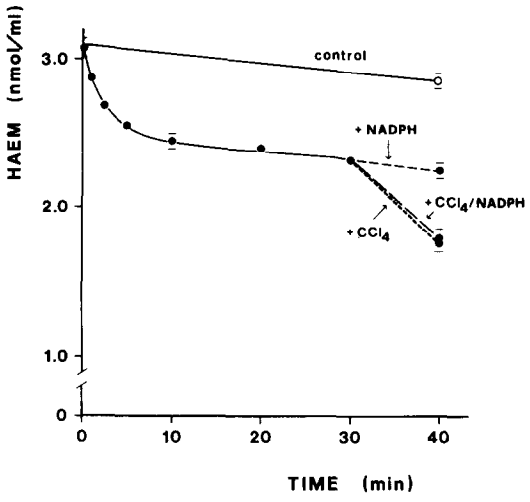

Fig. 4. Haem loss during metabolism of limiting concentrations of CCl_4 by microsomal cytochrome P-450. Experimental conditions and components were as for Fig. 3 but for the initial concentration of CCl_4 in the incubation mixture (15 μM). Control incubation contained NADPH and no CCl_4. As indicated, in 3 parallel incubations additional NADPH (2 μM) and/or CCl_4 (2 μM) were injected at 30 min. At the indicated times 0.8 ml of the incubation mixture were used for the pyridine/haemochrome reaction. Values indicated are mean ± SD of 3 determinations.

DISCUSSION

The metabolic activation of CCl_4 to reactive intermediates by the microsomal cytochrome P-450 system is an essential prerequisite for the hepatotoxicity produced both *in vivo* and *in vitro* by this toxic agent [38, 39]. It has been known for some time that during CCl_4 metabolism cytochrome P-450 itself, but not NADPH-cytochrome P-450 reductase, the other

Table 5. Partition ratio between metabolic turnover of CCl_4 and haem inactivation events during suicidal activation of CCl_4 by microsomal cytochrome P-450

CCl_4 (nmol/ml)	Haem (nmol/ml)			Partition ratio $\left(\frac{CCl_4 \text{ metabolized}}{\text{cyt. P-450 lost}}\right)$
	initial*	final†	lost‡	
0	3.05	2.95	—	—
10	3.05	2.56	0.39	26.5
15	3.05	2.39	0.56	26.8

* Total initial haem concentration was actually 3.05 ± 0.03 nmol/ml, including 2.40 nmol cytochrome P-450 haem/ml.

† Values indicate microsomal haem present at 20 min incubation when all CCl_4 has been metabolised.

‡ Figures indicate CCl_4-dependent loss of cytochrome P-450 haem after correction for the loss due to NADPH at 20 min (0.10 nmol/ml).

Anaerobic incubations contained 1 mg/ml microsomal protein (2.40 nmol cytochrome P-450/mg, from liver of Aroclor-pretreated rats) and 1 mM NADPH in 2 ml 0.1 M Na_2HPO_4 buffer, pH 7.4 in presence of the usual oxygen scavenging system. At time 0 CCl_4 was added to achieve the indicated initial concentrations and start the reaction. The incubation was terminated at 20 min when residual haem in the incubation mixture was determined by the pyridine/haemochrome reaction.

important component of the microsomal monooxygenase system, is inactivated [40, 41] and it is now largely accepted that cytochrome P-450 is the site of CCl_4 activation [42, 43]. CCl_4-induced lipid peroxidation of the endoplasmic reticulum and covalent binding of CCl_4 metabolites to microsomal lipids and proteins have both been suggested as the mechanisms responsible for CCl_4-dependent destruction of cytochrome P-450 *in vivo*, their relative contribution being dependent upon the experimental conditions [38]. During the anaerobic incubation of NADPH- or sodium dithionite-reduced liver microsomes with CCl_4, cytochrome P-450 can still be destroyed, even in the absence of lipid peroxidation [4–6]. Since the loss of the haemoprotein was accompanied by an equivalent loss of microsomal haem, measured by the pyridine/haemochrome method, it was suggested that cytochrome P-450 may be damaged by a direct attack of CCl_4 reactive metabolites on its prosthetic group [6]. The parallel and almost equimolar loss of cytochrome P-450 and haem observed in the present study (Fig. 1) is consistent with this hypothesis. We have now found that when the microsomal haem was assayed by an alternative technique, involving stoichiometric conversion of haem to protoporphyrin IX and measurement of porphyrin fluorescence, the loss of haem was accompanied by equimolar loss of porphyrin fluorescence (Table 1). This indicates that the tetrapyrrolic system of the haem moiety has been irreversibly modified by the attack of CCl_4 metabolites.

This fluorescence technique of haem estimation is not subjected to interference by high concentrations of CO—like the conventional pyridine/haemochrome reaction—so that the protective effect of CO on CCl_4-dependent haem loss could be investigated. The strong inhibition of the porphyrin loss observed with high concentrations of CO is consistent with the hypothesis that CCl_4-dependent haem destruction is a "suicidal" process requiring the interaction of CCl_4 with a reduced free haem iron [21], probably because the haem iron is involved in enzymatic or chemical transfer of electrons to CCl_4. Thus haem is not only the target but also the site of CCl_4 activation in cytochrome P-450. This view is also supported by the additional finding now reported that haem itself can catalyse its own CCl_4-dependent suicidal destruction in a purely chemical system, a rapid process which again involves loss of the porphyrin ring and depends upon a reduced haem iron and the presence of the reductant, sodium dithionite. No loss of volatile radioactivity was detected when bridge-labelled [^{14}C]-haem was incubated reductively with CCl_4, suggesting that, if the loss of porphyrin fluorescence is due to fragmentation of the tetrapyrrolic system, conversion of the bridge carbons or other labelled carbons to volatile products such as CO [15] did not occur.

The formation of a difference spectrum with a maximum at 454 nm is known to occur on addition of CCl_4 to anaerobically reduced rat liver microsomes [2]. This spectral change has been tentatively assigned to a ligand complex of the reduced haem iron with dichlorocarbene [35], for which more direct evidence has recently been obtained [18]. The formation of stable iron–porphyrin complexes upon reaction of CCl_4 with Fe(II)-tetraphenyl porphyrin in presence of a reducing agent has been known for some time [44]. The almost complete inhibition of this spectral change by DMB in the present study provides additional evidence for the carbene nature of the ligand. A potentially critical role of this species in cytochrome P-450 destruction and other aspects

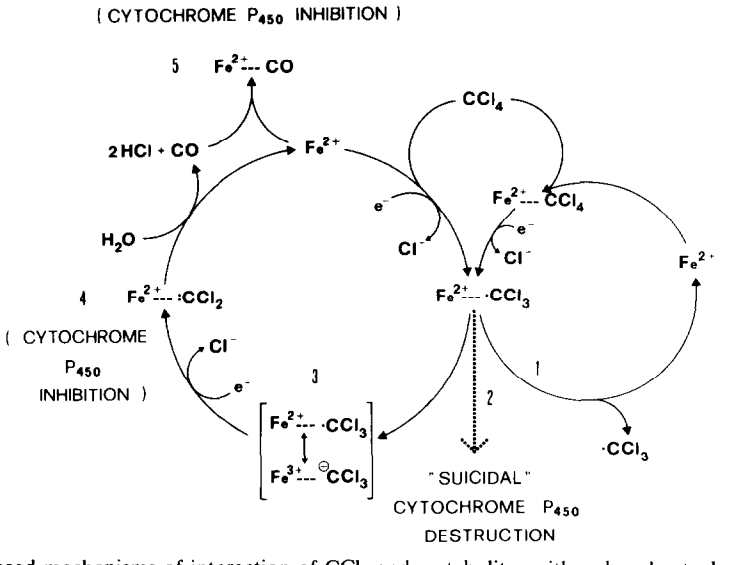

Fig. 5. Proposed mechanisms of interaction of CCl_4 and metabolites with reduced cytochrome P-450. Note that one electron reduction of CCl_4 produces $\cdot CCl_3$ and this can either be released from cytochrome P-450 (1), or irreversibly inactivate the cytochrome (2), or finally undergo a second one electron reduction leading to formation of $:CCl_2$ (3). This, while bound to the cytochrome acts as a reversible inhibitor (4) and on hydrolysis produces CO which can also bind the cytochrome (5). Fe^{2+} = reduced cytochrome P-450, Fe^{3+} = oxidised cytochrome P-450.

of CCl_4 toxicity has been proposed [45, 46]. The suggestion has also been put forward that the haem moiety of the cytochrome might be alkylated by $:CCl_2$ [6]. However, if $:CCl_2$ were to play a significant role in the process, one would expect that its trapping by DMB should have resulted in some protection against haem loss, and this was not found. We conclude therefore that the reactive species responsible is more likely to be $\cdot CCl_3$, the product of one electron reduction of CCl_4.

$:CCl_2$ hydrolyses spontaneously in solution to give CO, and the rate of its dissociation from haem and subsequent hydrolysis appeared to be affected by the apoprotein of cytochrome P-450. Our results showed an increased stability of the carbene–cytochrome P-450 complexes in microsomes from PB- and Aroclor-pretreated rats but not from β-NF-pretreated animals (Table 3). A possible explanation may be a higher affinity of these forms of cytochrome P-450 for the carbene, thus "discouraging" its dissociation from haem and the subsequent hydrolysis. A role of the apoprotein in modulating the release of carbene has also been suggested by Wolf *et al.* [35].

A good correlation was found between anaerobic formation of the two metabolites of CCl_4, CO and $:CCl_2$, and haem loss when liver microsomal preparations from rats pretreated with various inducers of cytochrome P-450 (Tables 3 and 4) were compared The higher loss of haem observed with PB microsomes in the present study is consistent with the observation that a rat cytochrome P-450 isoenzyme specifically induced by PB appears to be selectively responsible for and destroyed by the *in vivo* formation of $\cdot CCl_3$ from CCl_4 [16, 17]. More direct evidence for a suicidal type of inactivation reaction has now been obtained by showing that the CCl_4-dependent destruction of the cytochrome observed *in vitro* under reductive conditions is time- and substrate-saturable and exhibits pseudo first-order kinetics. Using a limiting concentration of substrate an attempt has also been made to calculate the partition ratio between catalytic and suicidal events. Enzyme inactivation occurs on average approximately once every 26 catalytic cycles.

Figure 5 summarizes the various steps involved in the reductive metabolism of CCl_4 by microsomal cytochrome P-450 and related interactions of the products ($\cdot CCl_3$, $:CCl_2$ and CO) with the haem of the cytochrome. After a one electron reduction of CCl_4 the free radical $\cdot CCl_3$ is formed. This can either be released from the active site of the haemoprotein (1) or bind the same prosthetic haem moiety where it was formed, leading to suicidal destruction of the haemoprotein (2), or undergo further reduction producing $:CCl_2$ (3). The carbene will then interact with the reduced haem iron leading to reversible inhibition of cytochrome P-450 (4) or undergo hydrolysis to CO, which can also bind to reduced cytochrome P-450 haem, again resulting in cytochrome P-450 inhibition (5). The possibility that $:CCl_2$ might attack and bind covalently to protein nucleophiles has also been considered [44].

In conclusion, the evidence described in the present paper indicates that (i) haem is the primary target of the suicidal reductive activation of CCl_4 by microsomal cytochrome P-450 or by non-enzymic haem preparation (MHA), (ii) in both cases reduced haem iron with at least one free axial ligand position is required for haem loss and this is due to a structural change involving modification of its porphyrin tetrapyrrolic system, (iii) the free radical but not the carbene is likely to be the species responsible for cytochrome P-450 destruction, and (iv) the destruction of cytochrome P-450 by CCl_4 is a typical suicide reaction where probably the same molecule of haem responsible for CCl_4 activation is attacked (on average once every 26 catalytic cycles) by a CCl_4 reactive metabolite.

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