STUDIES ON THE MECHANISM OF SUICIDAL INACTIVATION OF CYTOCHROME P-450 BY CARBON TETRACHLORIDE

A thesis submitted to the University of Surrey by

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in partial fulfilment of the requirements for the award of the degree of Ph.D. in Biochemistry

Department of Biochemistry, University of Surrey, Guildford, Surrey, GU2 5XH, U.K. February 1989
to the memory of my father
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<th>Definition</th>
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<tbody>
<tr>
<td>AIA</td>
<td>2-allyl-2-isopropylacetamide</td>
</tr>
<tr>
<td>ALA</td>
<td>aminolaevulinic acid</td>
</tr>
<tr>
<td>ALA-S</td>
<td>5-aminolaevulinate synthase</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>:CCl₂-P-450</td>
<td>dichlorocarbene-cytochrome P-450 complex</td>
</tr>
<tr>
<td>CDE</td>
<td>2-chloro-1,1-difluoroethylene</td>
</tr>
<tr>
<td>CTE</td>
<td>2-chloro-1,1,1-trifluoroethane</td>
</tr>
<tr>
<td>CO-Hb</td>
<td>carbon monoxide-haemoglobin complex</td>
</tr>
<tr>
<td>CO-P-450</td>
<td>carbon monoxide-cytochrome P-450 complex</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>DDC</td>
<td>3,5-diethoxycarbonyl-1,4-dihydrocollidine</td>
</tr>
<tr>
<td>DMB</td>
<td>2,3-dimethyl-2-butenone</td>
</tr>
<tr>
<td>dpm</td>
<td>disintegrations per minute</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>e.s.r.</td>
<td>electron spin resonance</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FMN</td>
<td>flavin mononucleotide</td>
</tr>
<tr>
<td>G-6-P</td>
<td>glucose-6-phosphate</td>
</tr>
<tr>
<td>G-6-PD</td>
<td>glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>Hb</td>
<td>haemoglobin</td>
</tr>
<tr>
<td>Hb-P-450</td>
<td>haemoglobin-cytochrome P-450 complex</td>
</tr>
<tr>
<td>H.p.l.c.</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>MDA</td>
<td>malondialdehyde</td>
</tr>
<tr>
<td>MFO</td>
<td>mixed function oxidase</td>
</tr>
<tr>
<td>MHA</td>
<td>methaemalbumin</td>
</tr>
<tr>
<td>Microsome</td>
<td>microsomal fraction</td>
</tr>
<tr>
<td>NAD(H)</td>
<td>nicotinamide-adenine dinucleotide</td>
</tr>
<tr>
<td>NADP(H)</td>
<td>nicotinamide-adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>β-NF</td>
<td>β-naphthoflavone</td>
</tr>
<tr>
<td>PBN</td>
<td>phenyl-t-butyl nitrone</td>
</tr>
<tr>
<td>PB</td>
<td>phenobarbitone</td>
</tr>
<tr>
<td>PDE</td>
<td>protoporphyrin IX dimethyl ester</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
</tr>
<tr>
<td>TBA</td>
<td>tetrabutylammonium hydroxide</td>
</tr>
<tr>
<td>T.l.c.</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low density lipoprotein</td>
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ABSTRACT

1. The molecular mechanism and the reactive species involved in the inactivation of microsomal cytochrome P-450 during the reductive metabolism of carbon tetrachloride (CCl₄) were studied. Results obtained with spectrophotometric, fluorimetric and chromatographic methods and using strictly anaerobic conditions indicate that the prosthetic group of the cytochrome, haem, is both the site and the target of CCl₄ activation.

2. Stoichiometric losses of microsomal haem and cytochrome P-450 were observed when CCl₄ was incubated anaerobically with NADPH- or sodium dithionite-reduced rat liver microsomes. A rapid loss of haem was also observed during reductive incubation of CCl₄ with soluble haem preparations (methaemalbumin) in the absence of the apoprotein. In both cases the loss of haem was accompanied by an equimolar loss of its tetrapyrrolic structure and was prevented by carbon monoxide. Similar results were obtained using halothane as a suicidal substrate.

3. A dichlorocarbene-cytochrome P-450 ligand complex was found to be responsible for the difference spectrum obtained on addition of CCl₄ to anaerobically reduced rat liver microsomes. A molar extinction coefficient of 56.2 for this complex has been calculated. The carbene trapping agent 2,3-dimethyl-2-butene did not prevent,
however, the $\text{CCl}_4$-dependent loss of microsomal haem indicating that the carbene is not involved.

4. Inactivation of haem by $\text{CCl}_4$, both in microsomes and the non-enzymic system is a typical suicide reaction which follows saturable, pseudo first-order kinetics. A partition ratio of 26 was calculated between molecules of $\text{CCl}_4$ metabolised and molecules of cytochrome P-450 haem lost. The loss of haem in the chemical system is due to covalent binding of $\text{CCl}_4$ reactive metabolites in a 1:1 stoichiometry.
1. General Introduction

1.1. Background

Although nowadays clinical cases of carbon tetrachloride (CCl\textsubscript{4}) poisoning are rare, the interest in studying both the effects and the mechanisms of CCl\textsubscript{4} hepatotoxicity has increased tremendously over the past decades so that CCl\textsubscript{4} is probably the most investigated hepatotoxin. CCl\textsubscript{4} was introduced into clinical therapy by M.C. Hall in 1921 for treating patients with the intestinal parasite hookworm, a disease called anchilostomiasis (Albert, 1981). Following the discovery of its lethal hepatotoxic and nephrotoxic properties in these patients, the use of CCl\textsubscript{4} in human therapy was discontinued. The compound is now used as a solvent.

The critical event in the history of CCl\textsubscript{4} toxicity was the recognition that the primary event leading to CCl\textsubscript{4}-dependent liver injury is the biotransformation of the chemical to highly reactive metabolites or intermediates. However, despite the enormous amount of work devoted to clarify the relationship between CCl\textsubscript{4} metabolism and cell injury or death, the mechanism of CCl\textsubscript{4} toxicity is still unclear. Several different attractive hypotheses have been put forward trying to link the primary metabolic events with the subsequent toxic changes in cell function and morphology resulting eventually in cell death. The evidence supporting these hypotheses
has been reviewed several times (Rechnagel & Glende, 1973; Slater, 1982; Rechnagel, 1983a; Brattin et al., 1985). This impressive effort has gone beyond the field of interest of toxicology and the interest in understanding the mechanism of CCl₄ hepatotoxicity has stimulated the work of researchers in several other basic sciences such as molecular pathology, biochemistry and physiology, making CCl₄ the most widely used model hepatotoxin.

1.2. The hepatotoxicity of carbon tetrachloride

The morphological changes induced selectively in the liver by CCl₄ given by gavage or by other routes are typically centrilobular necrosis and fat accumulation in hepatocytes. These changes were described as early as 1936 by Cameron & Karunaratne following a single dose of CCl₄ in the rat. They are similar in several species such as man, monkey, rat, mouse, rabbit, guinea pig, hamster, cat, dog, sheep and cattle (Zimmerman, 1978). After a single oral dose, lipid accumulation can be observed within the first hour by electron microscopy and within three hours by the light microscope (Smuckler, 1975). Fat accumulation was found to be mainly due to a block of the secretion into plasma of hepatic triglyceride as very low density lipoproteins (VLDL) (Lombardi, 1966), as a consequence of the inhibition of protein synthesis induced by CCl₄ (Dianzani, 1979). Single-cell necrosis is observed within six hours and centrilobular
necrosis after 12 hours from dosing, resulting in a significant increase of some liver enzymes (aspartate and alanine aminotransferase, lactate dehydrogenase, etc.) in serum.

Early changes are observed in the endoplasmic reticulum (ER) by electron microscopy which include degranulation of ribosomes from the rough ER and dilation of the cisternae of the rough and smooth ER. They account for the marked depression of protein synthesis observed within one hour after CCl₄ administration (Smuckler et al., 1962). Later changes are observed in Golgi apparatus, mitochondria and lysosomes (Dianzani, 1954 and 1963).

Early biochemical changes were observed which also appeared to be localized selectively in the membranes of the ER. These included depression of protein synthesis, glucose-6-phosphatase activity, cytochrome P-450 content and associated monooxygenase activities, whereas no decrease of NADPH-cytochrome P-450 reductase, NADH-cytochrome c reductase or triglyceride biosynthesis was observed (Recknagel & Lombardi, 1961; Glende, 1972; Smuckler et al., 1962).

1.2.1. Biochemical mechanisms of CCl₄ hepatotoxicity

The overall sequence of CCl₄ hepatotoxicity has been usefully divided into three separate subsequent events: 1. the metabolic activation of CCl₄ and the formation of reactive species or
metabolites, namely the trichloromethyl radical (·CCl₃); 2. the secondary, critical changes at biochemical level; and 3. the pathological consequences constituting the scenario of CCl₄ liver injury.

The third point has just been discussed and the first will be considered in some detail later. It will be mentioned briefly, now, the secondary mechanisms which are generally thought to link the initial step of CCl₄ activation to the eventual signs of toxicity. They are the covalent binding of CCl₄ metabolites to cell macromolecules, the peroxidative degradation of membrane lipids and the impairment of Ca++ homeostasis.

1.2.1.1. Covalent binding

It has long been proposed that the toxicity of CCl₄ depends on the covalent binding of CCl₄ reactive metabolites to tissue macromolecules. Reynolds (1967) showed that within 2 hr after the oral administration of [¹⁴C]- or [³⁶Cl]-CCl₄ to adult rats the radiolabel was incorporated into lipids, proteins and acid-soluble constituents of their livers. Microsomal lipids seemed to be preferentially labelled. When newborn rats were used, the amount bound per gram of liver was reduced to less than half. The authors concluded that CCl₄ "is toxic to liver cells by virtue of chemical attack of its cleavage products on the lipid and protein components
of the endoplasmic reticulum". This hypothesis was supported later by other results (Uehleke et al., 1973; Uehleke & Werner, 1975; Gillette et al., 1974). Castro & Diaz Gomez (1972) found that liver and kidney microsomes had greater ability to activate $^{14}$C-CCl$_4$ than the respective mitochondrial or 105,000 g supernatant fractions. In vitro studies from the same laboratory showed that the irreversible binding of $^{14}$C from $[^{14}$C]-CCl$_4$ to liver microsomal lipids required NADPH, was significantly greater under anaerobic conditions and almost completely inhibited by CO (Villarruel et al., 1975). These and other results give support to the hypothesis that irreversible binding to cellular components is the major mechanism responsible for CCl$_4$-induced cell damage (Villarruel et al., 1977; Castro et al., 1972). However other studies seem to support the peroxidative decomposition of lipids as the critical secondary change responsible for CCl$_4$ toxicity.

1.2.1.2. Lipid peroxidation

A pro-oxidant action of CCl$_4$ was first reported in 1965 in rat liver homogenates (Comporti et al., 1965) and in microsome-supernatant fraction prepared from a rat liver homogenate (Ghoshal & Recknagel, 1965). An important advance came from the observation that NADPH was an essential cofactor since no induction of lipid peroxidation was promoted by CCl$_4$ in NADPH-unsupplemented microsomes
These latter authors showed that stimulation of lipid peroxidation, as measured by the production of malondialdehyde (MDA), in microsomal suspensions was proportional (correlation coefficient, r=0.82) to the square root of the CCl₄ concentration in the incubation mixture. They also found that the increased production of MDA was accompanied by a decreased glucose-6-phosphatase activity, and both effects were more pronounced when bromotrichloromethane (CBrCl₃) was used instead of CCl₄, suggesting that the bond dissociation energy for the production of the free radical was a critical factor in the stimulation of lipid peroxidation by haloalkanes. They concluded that both effects observed were secondary to homolytic bond fission of the halogenomethanes to free radical products (Slater & Sawyer, 1971a). The formation of ·CCl₃ has only recently been demonstrated unequivocally by electron spin resonance (e.s.r.) using spin trapping techniques (Poyer et al., 1980; Tomasi et al., 1980). Under aerobic conditions ·CCl₃ reacts rapidly with molecular oxygen giving the trichloromethyl peroxy-radical (CCl₃O₂⁻), a product even more reactive and probably a stronger initiator of lipid peroxidation than ·CCl₃.

The relationship, however, between CCl₄ activation and CCl₄-induced lipid peroxidation appeared to be a complex one. For example, CO - a potent inhibitor of cytochrome P-450-catalyzed
reactions, including CCl₄ reduction - did not prevent CCl₄-dependent lipid peroxidation in microsomes or reconstituted monoxygenase systems (Slater, 1972; Wolf et al., 1980). On the other hand, the potent lipid peroxidation inhibitor promethazine protected microsomes or isolated hepatocytes against the CCl₄-dependent decrease in glucose-6-phosphatase but not against the CCl₄-mediated loss of cytochrome P-450 (Poli et al., 1981). These and other results indicated that the effects of CCl₄ treatment may be the result of a combination of different damaging mechanisms like covalent binding, lipid peroxidation and impairment of Ca²⁺ homeostasis and that other secondary events like NADPH depletion, depression of protein synthesis, etc. may also be involved, as extensively discussed by Slater (1982).

1.2.1.3. Impairment of Ca²⁺ homeostasis

The observation that rat liver microsomes have significant ATP-dependent calcium sequestration activity (Moore et al., 1975) and that CCl₄ administration to rats depresses Ca²⁺ uptake by liver microsomes in these animals (Moore et al., 1976), promoted a new line of research into the mechanisms of CCl₄ toxicity. These authors suggested that disturbed endoplasmic reticulum calcium pump activity may have a critical role in the expression of CCl₄ hepatotoxicity. Further work in this line from another laboratory showed that
depression of microsomal calcium sequestration in vitro appeared to be correlated to CCl₄- and CBrCl₃-dependent lipid peroxidation (Lowrey et al., 1981). These authors found also that microsomal calcium sequestration could be rapidly and severely depressed both by covalent binding of CCl₄ in the absence of lipid peroxidation or by CCl₄-independent lipid peroxidation (Waller et al., 1983) and concluded that disturbed hepatocellular Ca⁺⁺ homeostasis could be a critical link between CCl₄ metabolic activation and toxicity.

This hypothesis (Recknagel, 1983b), however, was not supported by more recent work. Brattin & Waller (1984) found that 1 μM to 1 mM CCl₄ caused a progressive decrease in total cell Ca⁺⁺ of isolated hepatocytes; but this was mainly due to loss from the mitochondria, probably through a lipid peroxidation-independent mechanism (Albano et al., 1985). Moreover, high doses of CCl₄ (>1mM) caused an increase in cell Ca⁺⁺ and cytosolic free Ca⁺⁺ but both effects seemed to be unrelated to CCl₄ toxicity, leading the authors to conclude that an increase in cytosolic free calcium is unlikely to be important in the toxic action of CCl₄ (Brattin et al., 1985). So a critical role of the impairment of Ca⁺⁺ homeostasis in CCl₄ toxicity has yet to be unequivocally demonstrated.
1.2.2. The role of metabolism in carbon tetrachloride hepatotoxicity

An early critical breakthrough in the understanding of the mechanism of CCl₄ activation and toxicity came from the finding by Butler (1961) of chloroform (CHCl₃) in the expired air of dogs treated with CCl₄ and in incubations of homogenates from different mouse tissues with CCl₄. All tissues tested were able to reduce CCl₄ to CHCl₃, the liver being the most active and the skeletal muscle having the least activity. Butler showed that under his experimental conditions several non-enzymatic reducing agents such as reduced glutathione (GSH), cysteine and ascorbic acid were also able to catalyze the reaction. These fundamental observations indicated that CCl₄ was metabolized to chloroform both in vivo and in vitro leading this author to postulate the formation of the trichloromethyl radical by homolytic fission of the carbon-halogen bond and to suggest that this reaction underlies the hepatotoxicity of CCl₄. In 1966 Slater proposed that CCl₄ is activated in the ER to highly reactive free radicals, probably ·CCl₃, by symmetrical fission of a C-Cl bond (Slater, 1966). This free radical, in Slater's theory, may attack cell constituents resulting in irreversible changes of liver cell structure and function.

At about the same time this hypothesis for the mechanism of CCl₄ hepatotoxicity was put forward by another group (Recknagel &
Ghoshal, 1966). These authors suggested also that the toxic effects of $\text{CCl}_4$ on the liver were due to the formation in the ER of free radicals by homolytic cleavage of the $\text{CCl}_3$-$\text{Cl}$ bond and they gave this reaction the colourful name of "lethal cleavage". They suggested, however, that $\text{CCl}_4$ hepatotoxicity was the result of a peroxidative decomposition of structural lipids of the liver cell and subsequent formation of toxic lipid peroxides and hydroperoxides ("lipid peroxidation theory"), rather than to a direct attack of $\text{CCl}_4$-derived free radicals on protein functional groups, (Recknagel & Glende, 1973). In 1965 Comporti et al. had shown that $\text{CCl}_4$ induces lipid peroxidation in rat liver microsomes. Based on thermodynamic considerations, Gregory (1966) noted that the activation reaction responsible for the production of the postulated free radical was unlikely to be an homolytic cleavage, as initially proposed by Butler, and he proposed a one electron reduction instead, as shown in reaction (1):

\[
\text{(1) } \text{CCl}_4 + e^- \rightarrow \cdot \text{CCl}_3 + \text{Cl}^-
\]

Although early attempts to trap $\cdot \text{CCl}_3$ were not successful (Ingall et al., 1978), more recently Poyer et al. (1980) and Tomasi et al. (1980) reported the trapping of $\cdot \text{CCl}_3$ in liver microsomal fractions using the spin trap PBN. Similar results were also obtained by
Whatever the mechanism of CCl₄ activation and toxicity, it became clear that the toxic effects were strictly dependent on the prior metabolic activation of CCl₄ to reactive metabolites.

1.2.2.1. Aerobic metabolism of CCl₄

It has long been known that CCl₄ is metabolised in vivo to various products including CO₂, CHCl₃ and CCl₃CCl₃ (McCollister et al., 1951; Butler, 1961; Fowler, 1969). Seawright & McLean (1967) showed that CCl₄ is metabolised to CO also in vitro by NADPH-supplemented rat liver microsomes. More recently Shah et al. (1979) reported that phosgene (COCl₂) is a metabolite of CCl₄ produced by rat liver homogenate; this observation was confirmed by Kubic & Anders (1980) in hepatic microsomal fractions. These authors also showed that a) the oxygen atom of COCl₂ derives from molecular O₂ and b) the reaction is inhibited by CO, both observations suggesting the involvement of the cytochrome P-450-dependent monooxygenase system. Recently, a new reactive metabolite of CCl₄, the so called "electrophilic chlorine" (probably Cl⁺), was reported to be formed by aerobic incubation of rat liver microsomes and five different mechanisms were proposed for its formation (Mico & Pohl, 1983). The mechanism of COCl₂ and electrophilic chlorine formation
from $\text{CCl}_4$ was investigated further by this group in rat liver microsomes (Pohl et al., 1983). They found that the rate of $\text{COCl}_2$ formation increased when the $O_2$ concentration was decreased from 100 to 5%, and decreased again at $O_2$ concentrations below 5%. They suggested that after reductive dechlorination of $\text{CCl}_4$ to $\cdot\text{CCl}_3$, this free radical is trapped by $O_2$ to form $\text{CCl}_3O_2^-$. This reaction, which was catalysed by a PB-inducible form of cytochrome P-450, was called "reductive oxygenation". Another group had already suggested that $\text{CO}_2$ and $\text{COCl}_2$ were formed from $\text{CCl}_3O_2^-$, the free radical produced by the reaction of $\cdot\text{CCl}_3$ with $O_2$ (Packer et al., 1978).

1.2.2.2. Anaerobic metabolism of $\text{CCl}_4$

When $^{14}\text{C-CCl}_4$ was incubated anaerobically with liver microsomal fractions in the presence of NADPH, $\text{CHCl}_3$ was produced accompanied by covalent binding of radiolabel (Uehleke et al., 1973). Under similar conditions, using NADPH or sodium dithionite as a reducing agent, small amounts of CO were formed, probably as a product of a dichlorocarbene-cytochrome P-450 complex (Wolf et al., 1977). It had been reported (Uehleke et al., 1973) and confirmed (Cox et al., 1976; Wolf et al., 1977) that under anaerobic conditions $\text{CCl}_4$ forms a complex with cytochrome P-450 showing a Soret absorption peak at approx. 454 nm. Ahr et al. (1980) showed conclusively that a) CO is a true metabolite of $\text{CCl}_4$ and b) the reaction is dependent on
cytochrome P-450, as indicated by its inhibition by exogenous CO or metyrapone. They also suggested that CO formation is due to hydrolysis of dichlorocarbene and that CHCl$_3$ production results from hydrogen abstraction by the ·CCl$_3$ radical. This latter aspect was then confirmed by GC/MS analysis showing no deuterium incorporation into CHCl$_3$ (Kubic & Anders, 1981). The reductive metabolism of CCl$_4$ and other halogenated alkanes under different oxygen concentrations was studied by Nastainczyk et al. (1982). These authors concluded that significant reductive in vitro metabolism of these compounds may occur even at physiological dioxygen concentrations and proposed a reaction scheme with two subsequent one electron reductions forming a free radical first and then a carbanion.

These studies and those reported above (1.2.2.1.) indicate that both aerobic and anaerobic activation of CCl$_4$ (Fig. 1) occur in vitro and in vivo, their relative contribution to CCl$_4$ toxicity being dependent upon the $O_2$ concentration in the incubation, cell or tissue.

1.2.3. The role of cytochrome P-450 in the activation of carbon tetrachloride.

Following the realization of the critical role of metabolism for CCl$_4$ toxicity, a considerable amount of work was carried out in order to establish the intracellular site of CCl$_4$ activation. It was
Fig. 1. Oxidative and reductive metabolism of CCl₄ and consequent events responsible for CCl₄ toxicity.
suggested earlier that NADPH-cytochrome P-450 reductase was involved in \( \text{CCl}_4 \) activation (Slater & Sawyer, 1971b). However, based on studies carried out in their laboratory, as well as in others', Recknagel & Glende (1973) concluded that "the locus of the lethal cleavage is the mixed function oxidase system of enzymes involved in the metabolism of drugs and foreign compounds". Their conclusion was based on the following indirect evidence:

i. \( \text{CCl}_4 \) forms a type I binding spectrum with liver microsomes indicating an interaction between \( \text{CCl}_4 \) and cytochrome P-450 (McLean, 1967).

ii. \( \text{CCl}_4 \) is not hepatotoxic in newborn rats, which are known to contain low levels of mixed function oxidase activity (Dawkins, 1963).

iii. Pretreatment of rats with a small dose of \( \text{CCl}_4 \) protects the animals from a normally lethal dose of the compound (Dambrauskas & Cornish, 1970) due to inactivation of liver mixed function oxidase (MFO) activity by the small dose of \( \text{CCl}_4 \) (Glende, 1972).

Subsequent work with both liver microsomes (Reiner et al., 1972) and reconstituted systems (Wolf et al., 1980) indicated that hepatic cytochrome P-450 was the enzyme responsible for the biotransformation of \( \text{CCl}_4 \) to chloroform. A good qualitative correlation was found between \( \text{CCl}_4 \) activation and P-450 content, while no correlation between \( \text{CCl}_4 \) activation and cytochrome P-450
reductase could be demonstrated by Villarruel et al. (1977). A critical role of cytochrome P-450, but not of cytochrome P-450 reductase, in the activation of \( \text{CCl}_4 \) became apparent also from the findings of Sipes et al., (1977) and Masuda & Murano (1978). More recently it was shown that \( \text{CCl}_4 \) can be activated to \( \cdot\text{CCl}_3 \) by a specific 52,000 dalton form of cytochrome P-450 in a reconstituted system containing the purified cytochrome, NADPH-cytochrome P-450 reductase, NADPH and \( \text{CCl}_4 \) (Noguchi et al., 1982a).

The role of cytochrome P-450 in the activation of \( \text{CCl}_4 \) has been investigated extensively and it is now well established both in vivo and in vitro.

1.3. The structure and function of the cytochrome P-450 system.

The haemoproteins of the cytochrome P-450 group are a family of isozymes with distinct, although overlapping, substrate specificity and properties. Cytochrome P-450, a microsomal pigment which in its reduced form binds carbon monoxide showing in the difference spectrum a typical absorption peak at approximately 450 nm (Kingenberg, 1958), is the terminal oxidase of the liver enzymatic system responsible for the monooxygenation of a large number of endogenous and exogenous substrates, such as steroids, fatty acids, lipophilic drugs, etc. These enzymes are largely distributed in nature, being found in most animal tissues and also in plants and
microorganisms. They catalyze an impressive number of different reactions including aliphatic and aromatic hydroxylation, N-oxidation, sulphoxidation, epoxidation, N-, S-, and O-dealkylation, peroxidation, deamination, desulphuration. A common feature underlying these reactions is the ability to convert oxygen to a more reactive species which is inserted into a poorly water-soluble substrate.

Some cytochrome P-450 isoenzymes can also function as a reductase catalyzing the reduction of haloalkanes (including CCl$_4$), azo and nitro groups, N-oxides and epoxides, or as an oxidase (White & Coon, 1980). The general enzymatic reactions catalyzed by cytochrome P-450 are reported in Fig. 2.

It is now well established that in all forms of this family of enzymes, the prosthetic and active group of the enzyme, protohaem, is bound to the protein part of the cytochrome (apoprotein) through a thiolate anion ($S^-$) linkage from a cysteine residue. Different types of cytochrome P-450 have apoproteins which are determined genetically and therefore differ in molecular weight, amino acid composition and antigenicity (Lu & West, 1980). Two other components of the cytochrome P-450 system, the flavoprotein NADPH-cytochrome P-450 reductase (an FMN- and FAD-containing protein) and cytochrome b$_5$ provide electrons to the cytochrome, probably through an interaction with the apoprotein of cytochrome P-450.
monooxygenase: $\text{RH} + \text{NADPH} + \text{H}^+ + \text{O}_2 \rightarrow \text{ROH} + \text{NADP}^+ + \text{H}_2\text{O}$

oxidase: $\text{NADPH} + \text{H}^+ + \text{O}_2 \rightarrow \text{NADP}^+ + \text{H}_2\text{O}_2$

reductase: $\text{e}^- + \text{RX} \rightarrow \text{R}^+ + \text{X}^-$

Fig. 2. Enzymatic activities of cytochrome P-450.
1.4. Mechanisms of "suicidal" degradation of cytochrome P-450

The chemical reactivity of a toxic metabolite is a most important factor in determining its fate within the cell. In general, the higher the chemical reactivity of the metabolite, the nearer to the site of its formation (usually an enzyme active centre) it will interact, either with some cell constituents, or even the actual activating enzyme. On the contrary, metabolites with low chemical reactivity are expected to be able to leave the enzyme or even the cell and react with distant target tissues. A number of substrates of cytochrome P-450 which fall into the first group are able to bind either the apoprotein or the haem prosthetic group of the cytochrome resulting in its irreversible inactivation. These compounds are usually known as "suicide" inhibitors or substrates of cytochrome P-450, although several alternative names have been used such as "mechanism-based enzyme inhibitors" or "enzyme-activated inhibitors" (De Matteis, 1987).

Since (i) more than one metabolite may be produced from a "suicide" substrate and (ii) even highly reactive metabolites (intermediates) can react with nucleophilic centres of macromolecules other than cytochrome P-450, not every single catalytic cycle results necessarily in "suicidal" inactivation of the haemoprotein. For some suicide substrates the partition ratio
between metabolic events and inactivation reactions has been calculated exactly (Loosemore et al., 1981).

Several extensive reviews on "suicide" substrates of cytochrome P-450 and their mechanisms of action are available (Ivanetich et al., 1978; Ortiz de Montellano & Correia, 1983; De Matteis, 1987). These compounds can be divided into two main classes according to their possible targets within the cytochrome: the protein and the prosthetic haem group. These two mechanisms of inactivation will now be discussed briefly with some examples.

1.4.1. Covalent binding to the apoprotein

Some "suicide" substrates, including carbon disulphide (CS$_2$), when administered to animals gave a rapid and significant decrease of drug-metabolizing enzymes in the liver (Freundt & Dreher, 1969) and a loss of cytochrome P-450 which was not accompanied by an early parallel loss of microsomal haem (Bond & De Matteis, 1969; De Matteis & Seawright, 1973), thus suggesting a primary effect on the apoprotein. These changes, due to their rapid onset, cannot be accounted for by inhibition of protein synthesis. Pretreatment of animals with PB gave a significantly higher loss of enzyme and also increased CS$_2$ metabolism to CO$_2$. In vitro incubation of CS$_2$ with NADPH-supplemented liver microsomes also resulted in cytochrome P-450 loss.
These and other results overall indicate that metabolism of \( \text{CS}_2 \) is responsible for the suicidal inactivation of the haemoprotein. With \( \text{CS}_2 \), a loss of microsomal haem has also been reported (Bond & De Matteis, 1969), but this is probably due to a stimulatory effect of \( \text{CS}_2 \) on physiological haem catabolism. Chloramphenicol is another example of a suicide substrate of cytochrome P-450 acting upon the apoprotein. Metabolites of chloramphenicol covalently bind to the apoprotein (mainly to lysine residues) resulting in the inability of the haem prosthetic group to be reduced by NADPH-cytochrome P-450 reductase (Halpert et al., 1985).

1.4.2. Covalent binding to the haem

Two suicide substrates which interact specifically with the haem have been particularly investigated; these are 2-allyl-2-isopropylacetamide (AIA) (and other unsaturated compounds) and 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC). Both compounds induce experimental hepatic porphyria and are powerful inducers of 5-aminolaevulinate synthase (ALA-S), the rate-limiting enzyme of haem biosynthesis. The activation of these compounds by cytochrome P-450 results in the alkylation of the prosthetic haem moiety and the formation of so called "green pigments". This reaction leads to irreversible inactivation of the haemoprotein and also to feedback stimulation of ALA-S and consequent induction of porphyria (De

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The mechanism of interaction with cytochrome P-450 haem, however, is different for the two compounds. It is now known that AIA, and other unsaturated compounds, are activated to reactive metabolites which alkylate one of the pyrrole nitrogens of the haem forming N-monosubstituted protoporphyrin products. With DDC a simple methyl group of the drug and not the entire compound (like for the unsaturated compounds) binds to one of the nitrogens forming N-methyl protoporphyrin, a product which is a selective and powerful inhibitor of ferrochelatase (De Matteis et al., 1980). Ortiz de Montellano et al. (1981) characterised by NMR studies all four structural isomers of N-methyl protoporphyrin.

1.4.3. The carbon tetrachloride-dependent inactivation of cytochrome P-450

A decreased activity of microsomal enzymes catalyzing the oxidative N-demethylation of dimethylaniline was first reported in rat liver following the administration of CCl$_4$ (Smuckler et al., 1967). An associated decrease in the content of cytochrome P-450, but not in that of cytochrome b$_5$, was also found by these authors, suggesting a specific action of the hepatotoxin on cytochrome P-450. The CCl$_4$-dependent loss of cytochrome P-450 and cytochrome P-450-associated enzymatic activities was confirmed by other studies and
it was attributed to peroxidative degradation of the microsomal membranes (Reiner et al., 1972; Recknagel & Glende, 1973).

Sasame et al. (1968) described in some detail the CCl₄-dependent in vivo destruction of rat liver cytochrome P-450. These authors showed that the induction of the drug-metabolizing system in these animals increased significantly the loss of haemoprotein. They reported that, within 3 hours after CCl₄ administration, more than 70% of the cytochrome P-450 was destroyed in liver microsomes of phenobarbital-treated rats. It was concluded by the authors that the decrease in cytochrome P-450 was not due to ER or liver cell destruction since the other microsomal protein NADPH-cytochrome c reductase was not decreased in liver microsomes, an observation subsequently confirmed also by in vitro studies (Ota et al., 1975). The authors suggested also that lipid peroxidation was not responsible for cytochrome P-450 destruction since ethanol, which was known to increase lipid peroxidation in microsomes, did not decrease the cytochrome P-450 content.

In another study Castro et al., (1968) showed that antioxidants did not protect from CCl₄-induced cytochrome P-450 loss, suggesting that lipid peroxidation was not involved. A lack of protection by antioxidants against the destructive effects of CCl₄ both in vivo and in vitro on microsomal drug metabolism was also reported by Dingell & Heimberg (1968).
Based on these and other results an alternative, lipid peroxidation-independent mechanism was proposed for the in vivo CCl₄-induced destruction of liver microsomal cytochrome P-450 (de Toranzo et al., 1975). According to this hypothesis the destruction of cytochrome P-450 is mediated by the direct attack of CCl₄ reactive intermediates onto the haemoprotein.

The early observations that in incubations where lipid peroxidation was prevented by the presence of EDTA (Uehleke et al., 1973) or by the absence of oxygen (anaerobiosis) (Glende et al., 1976; Recknagel et al., 1977) CCl₄ was still activated to reactive metabolites in the absence of any loss of cytochrome P-450 or glucose-6-phosphatase seemed to indicate that lipid peroxidation was responsible for cytochrome P-450 destruction by CCl₄. In agreement with this hypothesis was the observation that lipid peroxidation in liver microsomes resulted in a decreased cytochrome P-450 content even in the absence of CCl₄ (Levin et al., 1973).

The role of CCl₄-dependent lipid peroxidation in the decrease in glucose-6-phosphatase activity but not cytochrome P-450 content has been confirmed (Poli et al., 1981). It has been shown in several laboratories (Yamazoe et al., 1979; de Groot et al., 1980; Masuda, 1981), however, that even under anaerobic conditions in the absence of lipid peroxidation the activation of CCl₄ leads to marked loss of cytochrome P-450. Therefore, as stated by the authors themselves
(Recknagel, 1983a), the earlier conclusion that cytochrome P-450 was essentially unaffected by CCl₄ activation in the absence of lipid peroxidation (Recknagel et al., 1977) was in error and probably due to the presence of CCl₄ in control incubations. It is now generally accepted that reductive activation of CCl₄ in vitro under anaerobic conditions is associated with rapid loss of microsomal cytochrome P-450.

A considerable amount of data indicates that metabolic activation of CCl₄ leads both in vivo and in vitro to direct inactivation of the cytochrome's haem moiety, resulting in a decrease in microsomal cytochrome P-450 content and associated enzymatic activities. The evidence for this mechanism will now be reported briefly.

1.4.4. The CCl₄-dependent loss of cytochrome P-450 haem.

Following the observation by Smuckler's group of the CCl₄-dependent loss of hepatic microsomal cytochrome P-450, several authors confirmed and extended their results both in vivo (Vainio et al., 1976) and in vitro, including extrahepatic tissues (Chen et al., 1977; Castro et al., 1972). Green et al. (1969) and Levin et al. (1972) showed that the loss of the haemoprotein was accompanied by a parallel decrease of microsomal haem. The latter authors found that CCl₄ administration was responsible for the degradation of microsomal haem into (uncharacterized) breakdown products, thus
resembling in some respects the effect of 2-allyl-2-isopropylacetamide (AIA) (De Matteis, 1971). The haem loss with CCl₄, however, was greater than that observed with AIA, was accompanied by a decrease in microsomal protein and did not result in the formation of "green pigments" (Levin et al., 1972). More recently Guzelian & Swisher (1979) investigated the mechanism of the CCl₄-dependent degradation of cytochrome P-450 [¹⁴C]-haem in rats pretreated with 5-amino[5-¹⁴C]laevulinic acid ([5-¹⁴C]-ALA), a precursor which labels bridge carbons of haem. They concluded that CCl₄ accelerated catabolism of haem through non-carbon monoxide-forming mechanisms.

The mechanism of the inactivation of cytochrome P-450 by CCl₄ was investigated in vitro by de Groot & Haas (1980, 1981) using anaerobic incubations containing NADPH- or sodium dithionite-reduced rat liver microsomes. These authors reported that: (i) in presence of NADPH/CCl₄ or dithionite/CCl₄ the microsomal cytochrome P-450 content declined rapidly and low amounts of CO were formed; (ii) the decrease of cytochrome P-450 was always accompanied by an almost equimolar loss of microsomal haem; (iii) both cytochrome P-450 inactivation and haem loss were significantly prevented by metyrapone, an inhibitor of cytochrome P-450; and (iv) pretreatment of rats with PB markedly enhanced the loss of the haemoprotein and the formation of CO, whereas pretreatment with 3-methylcholanthrene
increased only CO formation. They concluded, in agreement with the mechanism proposed already by de Toranzo et al. (1975), that CCl₄ is activated by a PB-inducible form of cytochrome P-450 to reactive intermediates, probably ·CCl₃ or dichlorocarbene (:CCl₂), which attack directly the haem moiety of the cytochrome leading to irreversible inactivation of the haemoprotein.

1.5. Working hypothesis and objective of the study

The basic hypothesis of the present study was that CCl₄ is reductively activated to reactive metabolites/intermediates at the cytochrome P-450 active site, protohaem. These metabolites, depending on their chemical reactivity, can either leave the active site of the enzyme or undergo further metabolism or, alternatively, interact irreversibly with the prosthetic haem moiety, resulting in cytochrome P-450 destruction.

The aim of the present thesis is to describe the results of our studies on the mechanism of cytochrome P-450 inactivation during the reductive anaerobic metabolism of CCl₄ by rat liver microsomes.

The data presented demonstrate that the inactivation of cytochrome P-450 is a typical suicidal reaction where the prosthetic haem moiety is attacked and structurally modified by CCl₄ reactive metabolites, probably in a one to one stoichiometry. Spectral studies indicate that dichlorocarbene (:CCl₂), the product of the
two electron reduction of CCl$_4^-$, is probably not responsible for the loss of cytochrome P-450 haem, but only gives reversible inhibition of the haemoprotein. Finally, a non-enzymatic model for the CCl$_4^-$ dependent destruction of cytochrome P-450 haem was developed and will be described. Using this model the suicidal activation of CCl$_4^-$ by haem has been investigated and the resulting products of haem purified and partially characterised.

The experimental work on CCl$_4^-$ was carried out in the laboratories of the Department of Biochemistry of Surrey University, Guildford, Surrey (spectral studies and most studies on cytochrome P-450 inactivation) and the MRC Toxicology Unit, Carshalton (haem inactivation and radioisotopic/chromatographic studies).
2. Spectral changes induced in microsomes by CCl₄ and its metabolites.

2.1 INTRODUCTION

When compounds are added to microsomal preparations typical difference spectral changes are observed (Remmer et al., 1966; Schenkman, 1970). One group of compounds, which includes known substrates of the mixed function oxidase like hexobarbital, causes the appearance in the difference spectrum of an absorption peak at 388 nm and a trough at 420-422 nm. This spectrum has been called the type I spectral change and was attributed to an interaction between the substrate and cytochrome P-450. A second group of compounds, composed of basic amines like aniline, causes the appearance in the difference spectrum of an absorption peak in the Soret region and a trough at 390 nm. This spectral change has been termed as type II binding spectrum and was attributed to the formation of a complex between the haem of cytochrome P-450 and the substrate. The spectral change induced by a compound is not a prerequisite for metabolism by cytochrome P-450. It does indicate, however, an interaction between the compound and the haemoprotein (Schenkman, 1970).

It has been already mentioned that CCl₄ forms a typical type I binding spectrum with liver microsomal cytochrome P-450 (McLean, 1967). Interestingly, a type II spectral change has also been
described with microsomes obtained from rat adrenals (Castro et al., 1972). Under anaerobic reducing conditions CCl\textsubscript{4} added to microsomal suspensions causes also a difference spectral change which shows a peak of absorption at approximately 454 nm (Uehleke et al., 1973; Cox et al., 1976). This spectral change has been tentatively assigned to a ligand complex of the reduced haem iron with dichlorocarbene (:CCl\textsubscript{2}) (Wolf et al., 1977). These authors described the typical difference spectrum obtained after incubation of CCl\textsubscript{4} with sodium dithionite-reduced liver microsomes from phenobarbital-treated rats. The spectrum increased with time and showed a shift of the Soret peak with time from 460 to 454 nm. On addition of haemoglobin (Hb) to both test and reference cuvette the Soret peak decreased, the maximum shifted back to 460 nm and a sharp peak appeared at 419 nm which was attributed by the authors to a typical haemoglobin-carbon monoxide complex difference spectrum.

In the present work the spectral changes induced in rat liver microsomes by CCl\textsubscript{4} were further investigated in order to gain information on the metabolites of CCl\textsubscript{4} formed by cytochrome P-450 and their interaction with the prosthetic haem moiety, and evaluate their possible role in the inactivation of the haemoprotein.
2.2. MATERIALS AND METHODS

2.2.1 Chemicals and biochemicals

NADP⁺, catalase (EC 1.11.1.6), glucose oxidase (EC 1.1.3.4), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), human albumin, 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 from BOC (London, U.K.). Aroclor 1254 was from Monsanto Co. (St. Louis, MO, U.S.A) and a gift of Dr. C. Ioannides (University of Surrey, Guildford, U.K.). 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) and carbon tetrachloride from BDH Chemicals Ltd. (Poole, Dorset, U.K.). Sucrose was purchased from Merck (Darmstadt, West Germany). All other reagents were of analytical grade.

Haemoglobin (Hb) was prepared from rat blood using the method of Rossi-Fanelli and Antonini (1955). A total of approximately 8 ml of arterious blood was obtained by cardiac puncture from a Wistar Albino rat and heparinized. The blood was mixed gently and divided equally into two 10-ml test tubes, diluted 1:3 (v:v) in two volumes of 1% NaCl, mixed again and centrifuged at 1,200 rpm and 4°C for 10 min in a Beckman Model J-6B Centrifuge to separate the red blood cells (RBC) from plasma. Supernatant was discarded and the intact
RBC were resuspended in 10 ml 1% NaCl and centrifuged again at 1,200 rpm as above. This washing procedure was repeated twice before adding 8 ml water to each of the two tubes to haemolyse the RBC. The ruptured cell envelopes were then removed by centrifugation (4,000 rpm for 10 min) and the clear red solution of Hb was recovered and its concentration calculated by measuring the 418 nm peak obtained in the absolute spectrum of its complex with carbon monoxide, using the $\epsilon_{418}^\text{M} \cdot \text{cm}^{-1}$ 154. Then 0.5-ml samples of 3.5 mM Hb were flushed with oxygen-free nitrogen for a few minutes and stored at -20°C in 1-ml plastic vials.

Methaemalbumin (MHA), a water-soluble complex of haem with human albumin, was prepared as follows using the method of Tenhunen et al. (1968). Approximately 13 mg haemin (Sigma, Type I) were dissolved in 2.5 ml 0.1 N NaOH containing 12 mg Tris Base. To this solution 5 ml of a 2% (w:v) albumin (crystallized and freeze-dried) solution in twice distilled water were added and the pH was adjusted to 7.4 - 7.6 with 1 N HCl. The mixture was filtered through 2 sheets of Whatman N. 1 filter paper and the final volume adjusted to about 8 ml to obtain a final haem concentration of about 2.5 mM. The actual haem concentration in the filtrate was measured according to Paul et al. (1953) by adding 10 ul of the final solution to a few ml pyridine/0.3 M NaOH (15:27, v:v) before recording the reduced minus oxidised spectrum of this mixture in a Varian, Model Cary 219 or 2200, dual
beam UV-VIS spectrophotometer and using the $\epsilon_{\text{mM}^{-1}\cdot \text{cm}^{-1}}$ reported by Falk (1964a). One-ml samples were stored under nitrogen in plastic vials at -20°C, at which temperature they were found to be stable for years.

2.2.2. Pretreatment of animals and preparation of microsomes

Male Wistar Albino or Porton (Wistar-derived) rats (150-200 g) bred in the University of Surrey Animal Unit (Guildford, Surrey, U.K.) or the MRC Toxicology Unit Animal House (Carshalton, Surrey, U.K.) were numbered and kept four in each cage with water and Spratt's Laboratory Animal Diet I ad libitum for 3 to 7 days before treatment. Animals were weighed when provided and every day during treatment. In order to achieve induction of the liver cytochrome P-450 isoenzymes, animals were treated by intraperitoneal injections of phenobarbital (PB, 80 mg/kg body weight, daily for three days) or Aroclor 1254 (one single injection of 500 mg/kg body weight) or $\beta$-naphthoflavone ($\beta$-NF, 80 mg/kg body weight, daily for three days) or corn oil (controls, approximately 2.5 ml/kg body weight, daily for three days). Rats were starved overnight for approximately 18 hours before being killed by decapitation 24 hours after the last injection of corn oil, PB or $\beta$-NF, or four days after the single injection of Aroclor. After killing, the abdominal and thoracic cavities were opened and the liver was immediately perfused in situ
through the right atrium and the inferior vena cava with approximately 50 ml of ice-cold 0.9% (w:v) NaCl water solution to eliminate the blood. Then the liver was isolated, blotted dry with tissue and weighed. All the following procedures were carried out on ice. After removing the ilum, the parenchymal liver was weighed again, cut into small pieces with scissors and homogenised in three volumes of ice-cold 0.25 M sucrose using a 50 ml glass Potter type homogeniser with a teflon pestle (3 strokes at 3,500 rpm). The liver homogenates from rats which were identically treated were mixed together and then recentrifuged at 10,000 g (8,500 rpm) and 4°C for 20 min in a Beckman Model J2-21 Centrifuge using a 14 x 50 ml rotor. After centrifugation supernatants were mixed and 25-ml portions were pipetted into 25-ml ultracentrifuge screw tubes and centrifuged at 113,700 g (40,000 rpm) and 4°C for one hour in a Beckman Model L5-65 Ultracentrifuge with a 8 x 30 ml type 60 Ti rotor. After centrifugation the supernatant was discarded, tubes were wiped with tissue and the combined pellets were resuspended in a total of 200 ml of ice-cold 1.15% (w:v) solution of KCl in water using the 50-ml glass Potter homogeniser (3 strokes at 3,500 rpm) to wash microsomes. The various washed microsomal suspensions were then ultracentrifuged again as described above and the centrifuge tubes were then flushed with O₂-free nitrogen, stoppered and stored at -80°C. Alternatively, supernatants were discarded and the washed
pellet was resuspended in 0.1 M \( \text{Na}_2\text{HPO}_4 \) buffer, pH 7.4, containing 20\% glycerol, to obtain a final concentration equivalent to 2 g liver/ml. This final suspension was immediately used for cytochrome P-450, cytochrome b\(_5\) and protein determination or flushed with nitrogen and stored as described for the pellet. Stored under these conditions microsomal cytochrome P-450 was found to be remarkably stable, the specific content remaining unchanged for over six months.

### 2.2.3 Cytochrome P-450 determination

Specific content of cytochrome P-450 in final stock microsomal suspension was measured by the method of Omura and Sato (1964).

Two to five hundred ul of stock microsomal suspension were pipetted into 6 ml of 0.1 M \( \text{Na}_2\text{HPO}_4 \) buffer, pH 7.4 and mixed gently. Then 2.5 ml of this mixture were pipetted into each of two 3-ml glass cuvettes which were stoppered by a teflon cap. After recording the baseline between 500 and 400 nm, carbon monoxide was bubbled through the sample cuvette (1 bubble/sec for one min) and a few milligrams of \( \text{Na}_2\text{S}_2\text{O}_4 \) (sodium dithionite) were added to both cuvettes to reduce the microsomes. These were mixed gently and the difference spectrum was recorded a few times at about 1 min intervals in a Varian model Cary 219 or model 2200 spectrophotometer. Typical cytochrome P-450 spectra were obtained
with peaks at 448 or 450 nm depending on the rat treatment.

In some preparations a small shoulder at approx. 420 nm was observed, indicating that small amounts (usually <5% of total initial cytochrome P-450) of cytochrome P-420, the inactive haemoprotein derived from cytochrome P-450, were formed. Contamination of microsomal preparations with reduced haemoglobin or oxyhaemoglobin was determined by running a difference spectrum between 500 and 400 nm after addition of CO but before reduction by sodium dithionite and measuring any 418 nm peak formed. Usually the concentration of Hb in microsomes was found to be negligible (< 0.05 nmol/mg protein).

The largest difference in absorbance between 448/450 and 490 nm measured in the CO-reduced difference spectra was corrected, when necessary, for the baseline. The value obtained was used to calculate the concentration of cytochrome P-450 as follows:

\[
[Cyt. P-450] = \frac{\Delta E_{450-490} \times D}{91} \text{ mM}
\]

where D is the dilution of the stock suspension in the cuvette and 91 the millimolar extinction coefficient reported by Omura & Sato (1964). From this value and knowing the protein concentration, the
specific cytochrome P-450 content in microsomes (SC) was then calculated as follows:

\[
[Cyt. P-450] \text{(nmol/ml)} \\
\frac{\text{SC}}{\text{prot. conc. (mg/ml)}} \text{nmol/mg microsomal protein}
\]

(3)

In some cases the concentration of cytochrome P-450 was determined using only one cell (the cell used for the incubation) as described by De Matteis et al. (1977) by cooling it to 0°C and injecting into the incubation mixture 100 ul of a CO-saturated, oxygen-free solution of sodium dithionite (50 mg/ml of 0.1 M \( \text{Na}_2\text{HPO}_4 \) buffer, pH 9). The difference in absorbance between 450 and 490 nm in the reduced CO-absolute spectrum of this mixture was measured in a dual wavelength UV-VIS spectrophotometer model Perkin Elmer 356 and from this value the cytochrome P-450 content was calculated using the \( \epsilon_{91} \) reported above.

2.2.4 Cytochrome \( b_5 \) determination

Specific content of cytochrome \( b_5 \) in microsome was also measured by the method of Omura and Sato (1964). After diluting and processing the stock microsomal suspension as described above for the cytochrome P-450 measurement, a baseline was recorded between
450 and 400 nm with equal amounts of aerobic (oxidised) microsomes in test and reference cuvette. Then 50 ul of 12.6 mM NADH (0.25 mM final conc.) were added to sample cuvette to reduce cytochrome \( b_5 \) and a few spectra were recorded after 10 min. The difference in absorbance between 424 and 409 nm was measured and, after correction when necessary, for the baseline, used to calculate the concentration of cytochrome \( b_5 \) as follows:

\[
[Cyt. \ b_5] = \frac{\Delta E_{424-409} \times D}{185} \text{ mM}
\]

where \( D \) is again the dilution factor used and 185 the millimolar extinction coefficient reported by Omura & Sato (1964). From this value and knowing the protein concentration in microsomes the specific content of cytochrome \( b_5 \) in microsomes was calculated exactly as described above for cytochrome P-450.

2.2.5 Protein determination

Protein content in microsomes was measured using essentially the method of Lowry et al. (1951). The reagents were as follows (total volumes normally used in parentheses):

A) 2% (w:v) \( \text{Na}_2\text{CO}_3 \) in 0.1 N NaOH.

B) 1% (w:v) \( \text{CuSO}_4 \times 5 \text{H}_2\text{O} \).
C) 2% (w:v) Na/K tartrate.

D) Mixture of equal parts of B) and C) within 15 min before use (4 ml).

E) Mixture of 50 parts of A) and one part of D), to be used within 20 min (102 ml).

F) Mixture of equal volumes of phenol reagent and twice distilled water (10 ml).

The following three (1 test and 2 standard) stock solutions were prepared:

1) One hundred ul of the stock microsomal suspension were diluted 1:50 in twice distilled water to give a final vol. of 5 ml.

2) One hundred ul of 0.1 M Na$_2$HPO$_4$ buffer, pH 7.4, containing 20 % glycerol (the same used for suspending microsomes before storage) were also diluted 1:50 with twice distilled water.

3) One hundred ul of 20 mg/ml bovine serum albumin (BSA) and 100 ul of 0.1 M Na$_2$HPO$_4$ buffer, pH 7.4, were diluted with twice distilled water to a final vol. of 5 ml.

Samples containing increasing concentrations of microsomal protein were prepared by mixing 20-100 ul of stock solution 1) with 80-0 ul of 2), respectively, followed by addition of 0.4 ml twice distilled water to a final volume of 0.5 ml. A standard curve was prepared by mixing 0-100 ul of stock solution 3) with 100-0 ul of stock solution 2), respectively, followed by addition of 0.4 ml
twice distilled water. Test and standard (containing 0-40 ug BSA) solutions were then concurrently assayed in duplicate as follows. To each sample (test or standard) 2.5 ml reagent E) were added. The mixture was mixed and let to stand. After 10 min, 0.25 ml of reagent F) was added to each tube. The mixture was immediately mixed by whirling and the absorbance at 750 nm was read after 30 min in a Perkin Elmer model 550 or a Cecil model SP spectrophotometer. The protein concentration in test samples was finally calculated from the calibration curve obtained from the values of the BSA standard solutions.

2.2.6 Oxidised difference spectra of microsomes with CCl₄

Stock microsomal suspension (2.0 nmol cytochrome P-450/mg microsomal protein) was allowed to thaw and 125 ul of this suspension were added to 2 ml 0.1 M Na₂HPO₄ buffer, pH 7.4 and mixed gently to obtain a final concentration of 1 mg microsomal protein/ml. One-ml samples of this mixture were transferred to each of two 1-ml glass cuvettes and a baseline was recorded between 500 and 350 nm before the addition to the test cuvette of increasing amounts of CCl₄ in methanol. An equivalent amount of methanol was added to the reference cuvette. The following final concentrations of CCl₄ in the mixture were tested: 0.01, 0.11, 0.21, 0.31, 0.41, 0.51, 0.61 and 1.61 mM. A difference spectrum between 500 and 350 nm
was recorded after each CCl₄ addition.

In another experiment lauric acid (0.1 mM, final concentration) in methanol was added to both cuvettes before addition of various amounts of CCl₄ and recording the difference spectra.

2.2.7 Anaerobic incubations and oxygen-scavenging system

In order to achieve optimal reducing conditions and avoid peroxidative degradation of microsomes, all incubations were carried out, unless otherwise indicated, in oxygen-free 0.1 M Na₂HPO₄ buffer, pH 7.4. Strictly anaerobic conditions were routinely obtained as follows. The phosphate buffer to be used for the incubation mixture was bubbled through with oxygen-free nitrogen for at least one hour, before each experiment, followed by the addition of an oxygen-scavenging system comprising 600 U/ml catalase, 12.5 U/ml glucose oxidase and 60 mM D-glucose in the final incubation mixture. During each experiment the anaerobic buffer was kept on ice in a rubber-stoppered conical flask and flushed with oxygen-free nitrogen through two needles inserted in the rubber cap as described by Cooper et al. (1982). The anaerobic buffer so prepared and maintained is henceforth called "anaerobic buffer" or "usual anaerobic buffer".

Incubations were performed in rubber-stoppered glass cuvettes or tubes. The anaerobic buffer and, when possible, the reagents were
injected into these tubes or cuvettes using syringes previously flushed with nitrogen. When not in use, the glassware (tubes, syringes, small conical flasks, etc.) was kept in plastic bags under nitrogen atmosphere. All these precautions maintained the oxygen concentration in the incubation mixture normally below the lower limit of detection (about 0.2 μM) by the oxygen electrode (Hansatech, G.B.).

Usually, the stock microsomal suspension was kept on ice in rubber-stoppered tubes and individual samples, when needed, were taken by a syringe throughout the experiment. In some cases the total amount of microsomal suspension needed for the experiment was added initially to the anaerobic buffer and kept on ice throughout. Under these conditions the cytochrome P-450 content, measured at the beginning and the end of several experiments, was found to be particularly stable: no significant loss was observed after 4-5 hours in all cases.

The sodium dithionite solution was prepared fresh using 0.1 M Na₂HPO₄ buffer, pH 9, previously bubbled with oxygen-free nitrogen, in order to improve its stability. Only free-flowing samples of sodium dithionite with no odour of SO₂ were used as indicated by Falk (1964b).
2.2.8 Difference spectra of reduced microsomes with CCl₄

The usual procedure was the following. Identical amounts of microsomal protein were injected into each of two rubber-stoppered glass cuvettes containing 2.5 ml anaerobic buffer at 4°C, to obtain a final concentration of 1-1.2 mg/ml. When CO production from CCl₄ had to be studied, Hb was added to both cuvettes to a final concentration of 3 uM. After recording the baseline between 530 and 390 nm a sodium dithionite solution was injected into both cuvettes (11.4 mM final conc.) and a new baseline was run. In some cases this second baseline showed a peak or a trough at about 435 nm, possibly due to unequal reduction of some microsomal component in the two incubation mixtures. In these cases the baseline had to be re-run a few minutes later until the spectrum was satisfactorily flat. On a few occasions this could not be achieved and the incubation had to be discarded. At time 0, a few microlitres of 1 M CCl₄ in methanol were injected into the sample cuvette to a final concentration of 1.3 mM. The mixture was then gently mixed and spectra between 530 and 390 nm were recorded at 1, 3, 5, 10, 15, 20 and 30 min.

2.2.9 Concurrent determination of carbene and carbon monoxide

The amounts of :CCl₂ and CO formed from CCl₄ were concurrently measured in NADPH-reduced incubations from the difference spectrum
obtained between a sample cuvette (to which CCl₄ had been added) and a corresponding reference cuvette which contained no CCl₄, in the presence of 3 uM Hb, as described by Ahr et al. (1980). The CO present in the incubation mixture was quantitated from the difference in absorbance between the peak at 418/9 nm of CO-Hb and its isosbestic point at 411 nm, using a calibration curve constructed by serial additions of microlitre amounts of a CO-saturated water solution (approx. 1 mM) to a mixture containing both Hb and microsomes from PB-treated rats in presence of sodium dithionite or NADPH. The broad peak at 460 nm, which is thought to be due to a :CCl₂-cytochrome P-450 complex (:CCl₂-P-450), was employed to quantitate the carbene species, once the contaminating and endogenously formed CO had been displaced from cytochrome P-450 and trapped onto Hb. The 6mM⁻¹·cm⁻¹ 460-530 56.2 was used for calculation. This value was obtained, as described in more detail in 2.3.4, 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.

The typical incubation conditions used, unless otherwise indicated, were as follows. Identical amounts of liver microsomal protein from rats treated with PB were added to 1 ml anaerobic buffer in a test and a reference cuvette maintained at about 4°C in
the cooled cell-holder of a Varian dual beam spectrophotometer model Cary 219 or 2200. The anaerobic incubation mixture contained 1 mg microsomal protein, 3 uM Hb and a NADPH generating system comprising (final concentrations in parentheses) glucose-6-phosphate dehydrogenase (G-6-PD, 0.15 U/ml), glucose-6-phosphate (G-6-P, 1.9 mM) and NADP⁺ (0.12 mM), in 1 ml 0.1 M Na₂HPO₄ buffer, pH 7.4, containing the oxygen-scavenging system described in 2.2.7. At time 0, the reaction was started by injecting into the test cuvette a few microlitres of CCl₄ in methanol (to a final concentration of 1 mM). Incubation was at low temperature (4°C), so as to make the reaction slower and the formation of :CCl₂ and CO easier to read. This was achieved by cooling the circulating water bath of the spectrophotometer to 0-1°C with ice so as to maintain the temperature in both cuvettes around 4°C. The difference spectrum between the test and the reference cuvette was recorded at various incubation times. In some experiments microsomes from rats treated with corn oil (control) or various inducers of cytochrome P-450, as described in 2.2.2., were used.

When needed, the carbene-trapping agent 2,3-dimethyl-2-butene (DMB) was added (a few microlitres of a 2.1 M solution in methanol) to obtain a final concentration of 5 mM in both test and reference cuvette, before the addition of NADPH or sodium dithionite.

In other groups of experiments FMN (5-50 µM, final concentration)
or lauric acid (0.1 mM final concentration) was added to the incubation mixture before recording the baseline.

In one experiment FMN (0, 5, 10 and 50 uM, final concentrations) was added to 2 ml of the usual anaerobic buffer containing 1 mg microsomal protein/ml (2.07 nmol cytochrome P-450/mg) and 3 uM Hb, before dividing the mixture between two 1-ml cuvettes and recording the baseline. In this experiment the following NADPH generating system was also present in the incubation mixture: 0.4 U/ml G-6-PD, 5 mM G-6-P and 0.4 mM NADP+. CCl₄ (1 mM, final concentration) was added to the test cuvette and methanol to the reference cuvette before checking the baseline. At time 0, NADP⁺ was added to both cuvettes and the difference spectrum was recorded at time 1, 5, 10, 15 and 30 min. A similar experiment was carried out under the same conditions as the previous one but for the use, as the reducing agent, of NADPH instead of the NADPH-generating system, in both test and reference cuvette (approximately 1 mM, final concentration) and of 5 different final concentrations of FMN (0, 5, 10, 20 and 50 uM). In a third experiment carried out at 22°C in the absence of Hb and using sodium dithionite as the reducing agent, the effect of FMN on the combined carbene and CO-P-450 spectrum was investigated further.
2.2.10 Difference spectra of reduced haem with CCl₄

In some experiments haem, as MHA, was incubated under the same anaerobic conditions adopted with microsomes and the sodium dithionite-reduced difference spectrum between 530 and 390 nm was recorded on addition of CCl₄ to the test cuvette. Normally, 6.2 nmol haem (as MHA) and 2 umol sodium dithionite was added to each of two rubber-stoppered cuvettes containing 2 ml 0.1 M Na₂HPO₄ buffer, pH 7.4, made anaerobic as described in 2.2.7. At time 0, 1 umol CCl₄ in methanol was injected into the test cuvette; this was gently mixed and spectra were recorded after 1, 3, 5 and 10 min.

In some experiments 3 uM Hb (prepared as described in 2.2.1) was also present in both test and reference cuvette to trap the CO formed from CCl₄, as described in 2.2.9.

2.3. RESULTS

2.3.1. Oxidised difference spectra induced in rat liver microsomes by CCl₄

When liver microsomes from PB-treated rats were incubated with CCl₄ under aerobic conditions as described in 2.2.6, typical type I, dose-dependent difference spectra were obtained with a maximum at 388 nm and a minimum at 424 nm (Fig. 3). This was in agreement with previous reports (McLean, 1967).
The effect of type I substrates on the binding spectra induced by CCl₄ was also investigated in oxidised liver microsomes by including in the incubation mixture 0.1 mM lauric acid (dodecanoic acid). The spectra obtained in presence of lauric acid were qualitatively similar but markedly lower as compared to those obtained in the absence of lauric acid (results not shown). When the reciprocal of the difference in absorbance between the two wavelengths (388 and 424 nm) was plotted against the reciprocal of the substrate concentration in the usual double-reciprocal plot, Kₛ values of 0.27 and 0.71 mM were calculated in the absence and presence of lauric acid, respectively (Fig. 4).

These findings indicate that the binding of CCl₄ to the cytochrome P-450 binding site is competitively inhibited by lauric acid.

Further studies performed with lauric acid at concentrations up to 0.1 mM and aiming to investigate whether type I substrates have any effect on carbene complex formation and CO production by reduced microsomes gave negative results (data not shown). Higher lauric acid concentrations, however, could not be tested due to limitations in solubility.
Fig. 3. Difference spectrum of oxidised rat liver microsomes with CCl₄. See 2.2.6 for details.
Fig. 4. Double-reciprocal plot of type I difference spectra obtained on addition of CCl₄ to oxidised rat liver microsomes in the absence (A) and presence of lauric acid (B). See 2.2.6 for details.
2.3.2 Reduced difference spectra induced in rat liver microsomes by CCl₄ metabolites

When NADPH- or sodium dithionite-reduced liver microsomes were incubated at 4°C in the cooled cell-holder of the spectrophotometer with CCl₄ under anaerobic conditions, a difference spectrum showing an absorption peak at approximately 454 nm appeared (Fig. 5, A), as compared with appropriate controls not treated with CCl₄, and increased throughout the incubation period up to 60 min. A small, apparently constant peak was also present at about 422-4 nm in spectra from dithionite- but not NADPH-reduced incubations.

This spectrum (Fig. 5, A) is the result of two components. One component showing a peak at 460 nm was attributed to :CCl₂-P-450, the complex formed between CCl₄-derived dichlorocarbene (:CCl₂) and the haem of cytochrome P-450. Spectral evidence for attributing the 460 nm peak to a carbene metabolite of CCl₄ is reported in 2.3.3. The other component of the spectrum is due to the complex of CCl₄-derived and endogenously generated CO with reduced cytochrome P-450 haem (CO-P-450) and gives the classical 450 nm peak. This interpretation is based on the following observation.

In presence of Hb (Fig. 5, B) CO-P-450 is not demonstrable in the incubation mixture as the CO produced from CCl₄ is trapped as CO-Hb, giving a typical, sharp peak at 418/9 nm which can be used for quantitation as described in 2.2.9.
Fig. 5. Difference spectra of anaerobic sodium dithionite-reduced rat liver microsomes with CCl₄ in the absence (A) and presence (B) of haemoglobin. See 2.2.8 for details.
2.3.3 Spectral evidence for carbene formation

When the specific carbene trapping agent DMB was included (5 mM, final concentration) in the incubation mixture of both test and reference cuvette, a strong (> 95 %) inhibition of the 460 nm peak was observed, in the absence of Hb, in the difference spectrum obtained on addition of CCl₄ to dithionite- or NADPH-reduced liver microsomes from PB-treated rats (Fig. 6). On the contrary, no effect was found on the small 420 nm peak observed when dithionite but not NADPH was used as the reducing agent.

These results support the hypothesis that a carbene species is responsible for the 460 nm peak. This peak is henceforth referred to as the "carbene peak".
Fig. 6. Effect of the carbene-trapping agent 2,3-dimethyl-2-butene (DMB) on the difference spectrum of anaerobic, sodium dithionite-reduced rat liver microsomes with CCl₄. In both A and B, test and reference cuvettes contained 1.14 mg microsomal protein/ml (2.62 nmol cytochrome P-450/mg) and 0.9 mM sodium dithionite in 2.2 ml 0.1 M Na₂HPO₄ anaerobic buffer, pH 7.4. In A, 5 mM DMB was also present in test and reference cuvette. At time 0, CCl₄ (1 mM final concentration) was injected into the test cuvette and scans were recorded at 1, 3, 5 and 10 min. Incubation was at 4°C to slow down the reaction. After the last scan, 0.8 ml of test and reference incubation mixtures were used for haem determination (see 3.3.1.5).
2.3.4 Calculation of \( \text{CCl}_2 \text{-P-450} \) extinction coefficient

After incubation of PB microsomes with \( \text{CCl}_4 \) and sodium dithionite for 10 min at 4°C 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 inactivated by \( \text{CCl}_4 \), by determining in (B) the loss of haem due to incubation; b) the cytochrome P-450 available for further ligand formation - over that already complexed with either CO or \( \text{CCl}_2 \) - by adding to sample (B) saturating amounts of CO (1 bubble/sec for 1 min) at the end of incubation and determining the increase in CO-P-450 spectrum; c) the cytochrome complexed with \( \text{CCl}_4 \)-derived, endogenous CO, by subtracting from the \( \Delta A_{450-490} \) of the sample incubated without Hb (B) the corresponding \( \Delta A \) measured in the sample containing Hb (A); and finally, (d) the portion of haemoprotein complexed by carbene, by subtracting from the total cytochrome P-450 remaining after incubation the subfractions b) and c) above. Using this indirect method the \( \epsilon \text{mM}^{-1} \text{cm}^{-1} \) for \( \text{CCl}_2 \text{-P-450} \) was calculated to be 56.2.

2.3.5 Production of \( \text{CCl}_2 \) and CO and effect of inducers

The CO and \( \text{CCl}_2 \) formed during anaerobic incubation of different types of liver microsomes with \( \text{CCl}_4 \) is reported in Table 1.
Table 1. Effect of rat pretreatment on the CCl₄-dependent formation of :CCl₂ and CO by NADPH-reduced rat liver microsomes.

<table>
<thead>
<tr>
<th>Rat pretreatment</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>15</th>
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<tbody>
<tr>
<td>C</td>
<td>0.035</td>
<td>0.064</td>
<td>0.089</td>
<td>0.107</td>
</tr>
<tr>
<td>:CCl₂ (nmol/mg prot)</td>
<td>PB</td>
<td>A</td>
<td>β-NF</td>
<td></td>
</tr>
<tr>
<td>0.392</td>
<td>0.228</td>
<td>0.053</td>
<td>0.24</td>
<td></td>
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<tr>
<td>0.518</td>
<td>0.428</td>
<td>0.071</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
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<td>0.518</td>
<td>0.107</td>
<td>0.83</td>
<td></td>
</tr>
<tr>
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<td>0.546</td>
<td>0.142</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>CO</td>
<td>0.24</td>
<td>0.49</td>
<td>0.83</td>
<td>0.97</td>
</tr>
<tr>
<td>(nmol/mg prot) A</td>
<td>PB</td>
<td>β-NF</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>0.29</td>
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<td>1.80</td>
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</table>

Microsomes (1 mg protein) were incubated in each of two 1 ml cuvettes, a test and a reference cuvette being used for each microsomal preparation, in 1 ml anaerobic buffer containing 3 uM Hb and the NADPH generating system described under Materials and Methods. The reaction was started by injecting CCl₄ (1 mM) into the test cuvette. After incubation at 4°C for the indicated periods of time, the difference spectrum was recorded between 530 and 390 nm. :CCl₂ and CO were measured as their ligand complexes with cytochrome P-450 and Hb, respectively. In this experiment the initial cytochrome P-450 concentration was 1.5, 2.1, 2.5 and 1.6 nmol/mg protein in microsomes from control (C), PB-, Aroclor- (A) and β-naphtoflavone-(β-NF)-treated rats, respectively.
Microsomes from PB- or Aroclor-treated rats showed a greater ability to produce CO and \( \text{CCl}_2 \) than microsomes from control or \( \beta \)-NF-treated animals. The amount of CO formed is dependent on the hydrolysis of \( \text{CCl}_2 \), which is likely to occur rapidly once the latter species has become dissociated from cytochrome P-450. So, the higher the stability of \( \text{CCl}_2 \)-P-450, the lower will be the formation of CO. So, if one assumes that at any given time the total formation of \( \text{CCl}_2 \)-P450 is equal to the sum of \( \text{CCl}_2 \) and CO present in the incubation at that time, one can also consider the ratio of total amount of \( \text{CCl}_2 \) formed (i.e. \( \text{CCl}_2 \)-P-450 + CO) to that of CO produced as an index of the stability of \( \text{CCl}_2 \)-P-450. The higher the ratio, the greater will be the stability of the complex.

In Fig. 7 the effect of rat pretreatment on the stability of the \( \text{CCl}_2 \)-cytochrome P-450 ligand complex is reported. The ratio between \( \text{CCl}_2 \) + CO and CO produced has been plotted against time of incubation using different microsomal preparations to compare the stability of the complex in these different microsomes. With microsomes from rats treated with Aroclor or PB the stability of \( \text{CCl}_2 \)-P-450 was higher than that obtained with microsomes from \( \beta \)-NF- or corn oil-treated animals.
Fig. 7. Effect of rat pretreatment on the stability of the :CCl₂-cytochrome P-450 ligand complex. Data are those of the experiment of Table 1.
2.3.6 Effect of FMN on \( \text{CCl}_4 \)-dependent carbene formation and CO production in reduced microsomes

This was investigated in both NADPH- and dithionite-reduced microsomal incubations from PB-treated rats. When the NADPH-generating system was used the carbene peak at 460 nm appeared to be remarkably increased (1.6 and 5-6 fold with 5 and 50 uM FMN, respectively) in presence of FMN in a concentration-dependent fashion. In contrast, no significant change was observed in CO production (data not shown).

When NADPH was used as the reducing agent no effect on CO production was seen but an increase of the carbene peak was observed in this experiment in presence of FMN (1.6 fold with 5 uM FMN, when compared to an identical incubation in the absence of FMN).

In the third experiment where dithionite was used as the reducing agent two incubations, one in presence and one in absence of 5 uM FMN, were performed. As expected, both in absence and presence of FMN the peak showed the usual shift from 460 to 450 nm due to the increasing formation of the CO-cytochrome P-450 complex. In this experiment, however, the increasing effect of FMN on the carbene complex peak was small (about 15%).

The absolute spectrum of oxidised FMN (10 uM in 0.1 M \( \text{Na}_2\text{HPO}_4 \) buffer, pH 7.4) gives two large peaks at about 380 and 450 nm,
respectively. Both peaks disappear on reduction of FMN with a few milligrams sodium dithionite (results not shown). The lower effect observed in the third experiment, where dithionite was used, as compared to the previous ones where the NADPH-generating system or NADPH were used, could have indicated that the type of reducing agent used may play a role in determining the increase of the "carbene peak", possibly through a lower degree of reduction of FMN in the test cuvette which might be due to the presence of the substrate (CCl₄). So, the spectral changes induced by FMN might have been merely due to different degrees of reduction of FMN rather than to a true effect on the carbene complex formation.

If the increase in the 460 nm peak was due to increased carbene formation it would have been possible to inhibit the formation of the peak by trapping the carbene species thus preventing its binding to cytochrome P-450. To investigate this possibility the specific carbene-trapping agent DMB was included in the incubation mixture. These experiments are described below.

2.3.7 Effect of DMB on the "carbene spectrum" obtained with NADPH- or dithionite-reduced microsomes in presence of FMN

Two ml of anaerobic buffer containing 2 mg liver microsomal protein (2.6 nmol cytochrome P-450/mg) from PB-treated rats in presence or absence of 10 uM FMN were maintained anaerobically in
test and reference cuvette and a baseline was recorded before and after addition of NADPH or sodium dithionite. At time 0, CCl₄ was injected into the test cuvette to obtain a 1 mM final concentration. The cuvettes were mixed gently and scans were recorded between 530 and 390 nm at time 30 sec. and 3, 5 and 10 min. At time 12 min., microlitre amounts of Hb (4.5 uM, final concentration) were added to both cuvettes and, after gentle mixing, new spectra were recorded at 15, 20 and 25 min to investigate the formation of CO.

In these incubations FMN was only responsible for a significant increase in absorption in the region of the carbene peak when NADPH, but not dithionite, was used, thus confirming previous findings (see 2.3.6). When similar NADPH-reduced incubations containing also 5 mM DMB were performed, the carbene peak was almost absent, as expected, in the absence of FMN but the absorption at 450 nm was only approximately 50% inhibited in its presence. The CO-Hb peak, which appeared at about 418-420 nm on addition of Hb, was also inhibited by DMB both in presence and absence of FMN.

These results are difficult to interpret. They showed that DMB which inhibited almost completely the 460 nm peak in the absence of FMN could only partially do so in presence of FMN. This suggests that either the increase in the peak observed with FMN is not due to carbene but to a contribution from oxidised FMN or the carbene formed in excess cannot be trapped, for some reason, by DMB.
2.3.8 Reduced difference spectra of haem (as MHA) with CCl₄ in the absence and presence of Hb

Haem itself in a purely chemical system devoid of cytochrome P-450 apoprotein can interact, under reducing conditions, with CCl₄ undergoing rapid inactivation, probably by CCl₄-derived reactive intermediates. This will be described in more detail in another part of this thesis (see 3.3.3.1). It was reasonable, therefore, to speculate that CCl₄ might have been activated by haem in a similar way to that occurring with microsomes. If this was the case it might be possible to obtain with haem a difference spectrum similar to that given by reduced microsomes on addition of CCl₄.

When equal amounts of MHA were incubated in two cuvettes under the usual anaerobic conditions and in presence of sodium dithionite, as described in 2.2.7, a typical dose-dependent difference spectrum with a large peak at about 470 nm and a smaller peak at about 415 nm was observed on addition of a few microlitres of CCl₄ in methanol to the test cuvette. In Fig. 8 this spectrum is shown and a typical difference spectrum obtained with microsomes has been also reported for comparison.
Fig. 8. Difference spectra of anaerobic sodium dithionite-reduced haem, as MHA, (---) and rat liver microsomes (----) with CCl₄. See 2.2.10 and 2.2.8 for details on MHA or microsome incubations, respectively. For clarity, only the spectra recorded at time 5 min are reported.
In MHA incubations, as with microsomes, the ability of Hb to trap any CO present was used to investigate whether in a cytochrome P-450-free system CCl₄ could be transformed to CO. On addition or in the presence of Hb, both the wavelength and the size of the 470 nm peak were apparently unchanged, while a deep trough with a minimum at approx. 432 nm and a small, sharp peak at 418 nm were formed. In Fig. 9 the difference spectra obtained on addition of CCl₄ to dithionite-reduced haem before and after the addition of Hb are reported.

The small 418 nm peak observed in the presence of Hb was qualitatively different from the broader one observed in the absence of Hb (Fig. 8). Its wavelength corresponded exactly to that of the sharp, dose-dependent peaks formed on addition to the test cuvette of microlitre amounts of CO-saturated twice distilled water. For this reason and in analogy with the results obtained with microsomes, the peak was probably attributable to a CO-Hb complex formed by CCl₄-derived, endogenously generated CO. Very similar spectra were obtained when Hb was added to the anaerobic incubation mixture initially, before the addition of CCl₄ (results not shown).

In one experiment the effect of the carbene trapping agent DMB on this CCl₄-dependent, dithionite-reduced difference spectrum of haem was studied. In contrast to the results obtained with microsomes and reported above (see 2.3.3), the presence of 5 mM DMB (added in
Fig. 9. Difference spectra of anaerobic, sodium dithionite-reduced methaemalbumin with CCl₄ in the absence (—) and presence (---) of Hb. Both test and reference cuvette contained 2.48 nmol haem (as MHA)/ml and sodium dithionite (1 mM) in 2.5 ml anaerobic buffer. At time 0, CCl₄ (1 mM) was injected into the test cuvette and after 0.5, 3 and 5 minutes of incubation at 4°C a difference spectrum was recorded between 530 and 390 nm (—). At time 8 min haemoglobin (6.0 μM) was added to both cuvettes and new spectra were recorded at 11, 15 and 20 min (---). Finally 1, 3, 5 and 7 μl of a CO-saturated anaerobic buffer were injected into the test cuvette and a spectrum was recorded immediately after each addition (-.-).
methanol to the incubation mixture of both test and reference cuvette) did not appear to have any significant effect on the haem spectrum (results not shown).

2.4 DISCUSSION

It has been known for over twenty years that CCl₄ produces in microsomes a spectral change similar to that produced by hexobarbital (McLean, 1967), the so-called type I binding spectrum. This spectral change is characterized by a decrease in absorbance at about 420 nm and an increase around 390 nm (Remmer et al., 1966) and it is thought to be due to an interaction of the compound with the protein component of cytochrome P-450, resulting in a change of the spin state of the cytochrome (Sato & Omura, 1978). As reported by Schenkman et al. (1970) the type I spectral change was shown to parallel the enzyme activity and pretreatment of rats with PB, a well known inducer of some cytochrome P-450 isoenzymes, caused an equivalent increase of the type I spectral change. A typical, dose-dependent type I binding spectrum was also found in the present work on incubation of an oxidised microsomal suspension from PB-treated rats with CCl₄. This indicated that CCl₄ interacted, as expected, with the apoprotein part of cytochrome P-450.

In agreement with this interpretation was the observation that another type I substrate, lauric acid, inhibited the CCl₄ spectrum
competitively. It is possible that lauric acid added to the microsomal suspension before CCl₄, had occupied some of CCl₄ binding sites onto the cytochrome P-450 apoprotein, thus preventing in part, on addition of CCl₄, the CCl₄-protein interaction and the consequent formation of a binding spectrum.

From Lineweaver-Burk plots the calculated Kₛ value for the type I difference spectrum with aerobic microsomes and CCl₄ was 0.27 mM in absence of lauric acid and 0.71 in its presence. The Kₛ value obtained in the present study in absence of lauric acid with liver microsomes from PB-treated rats is lower than that reported by others using liver microsomal preparations from untreated rats (Cox et al., 1976) or PB-treated rabbits (Uehleke et al., 1973). The formation of the carbene spectrum, however, was not changed by lauric acid suggesting that binding of a type I substrate to anaerobic microsomes does not affect the reductive metabolism of CCl₄. It is possible that different cytochrome P-450 isoenzymes may be primarily responsible for CCl₄ reductive metabolism and CCl₄-induced type I spectral change. Another reason for the lack of effect of lauric acid on the carbene spectrum could be that binding of CCl₄ to the apoprotein is not required for, or at least is not a limiting step in CCl₄ activation. In agreement with this interpretation is the finding that lauric acid, unlike metyrapone, was also unable to inhibit the CCl₄-dependent loss of cytochrome P-
450 haem (results not shown).

The formation of a difference spectrum with a maximum at approximately 454 nm is known to occur on addition of CCl₄ to anaerobically reduced rat liver microsomes (Uehleke et al., 1973) and it has been confirmed by others (Cox et al., 1976; Wolf et al., 1977; Ahr et al., 1980). This spectral change had been tentatively assigned to a ligand complex of the reduced haem iron with trichloro carbanion (Ullrich & Schnabel, 1973). Further work (Wolf et al., 1977; Ahr et al., 1980) showed that the spectral change was due to two components: a ligand complex between the haem of cytochrome P-450 and dichlorocarbene (:CCl₂, a reactive metabolite which had been postulated to be formed after a two electron reduction of CCl₄ by cytochrome P-450) and a second ligand complex between carbene-derived CO and cytochrome P-450. Recently :CCl₂ has been conclusively identified as a reactive metabolite of CCl₄ (Pohl & George, 1983).

A difference spectrum similar, if not identical, to those reported by the authors cited above has also been observed in the present study. The almost complete inhibition of the spectral change by DMB observed in the present study (Fig. 6) provided additional evidence for the carbene nature of the ligand.

According to the present results and those on the loss of cytochrome P-450 haem presented elsewhere in this thesis (see
3.3.1), 4 major different pools of cytochrome P-450 may be envisaged to be present in the incubation mixture during the anaerobic reductive activation of \( \text{CCl}_4 \) by liver microsomes. Two of these pools, which have been already mentioned, are represented by the cytochromes forming a complex with either \( \text{CCl}_2 \) or CO, respectively. The third group comprises the forms of cytochrome P-450 unable to metabolise \( \text{CCl}_4 \) and therefore resistant to \( \text{CCl}_4 \)-dependent inactivation. The last pool includes the haemoprotein which has been attacked and irreversibly modified in its prosthetic group by \( \text{CCl}_4 \) reactive metabolites. This last aspect will be described later in the present thesis.

On this assumption and knowing the concentration of 3 of these components and that of total cytochrome P-450 haem present, the concentration of the fourth component, i.e. the portion of cytochrome P-450 haem complexed by the carbene, could be calculated. From this value and assuming a 1:1 carbene/cytochrome ratio in the complex, an extinction coefficient for the 460 nm peak of the \( \text{CCl}_2 \)-P-450 complex was tentatively derived. This \( \varepsilon \text{mm}^{-1} \cdot \text{cm}^{-1} \) value, however, was calculated indirectly and assuming that all possible forms of cytochrome P-450 haem has been accounted for. It should only be considered, therefore, an approximation which needs confirmation by more direct methods.

\( \text{CCl}_2 \) hydrolyses spontaneously in solution to form CO, and a
linear relationship has been found between $\text{CCI}_2$ and CO production with a correlation coefficient $r = 0.94$ (Ahr et al., 1980). The rates of CO production by PB-microsomes reported in the literature (1.4 and 2.4 nmol/mg protein/10 min according to de Groot & Haas (1981) and Ahr et al. (1980), respectively) are similar to those found in the present study (1.5 nmol/mg protein/10 min). The rate of dissociation of $\text{CCI}_2$ from haem and its subsequent hydrolysis to CO appear to be affected by the apoprotein of cytochrome P-450. When the production of $\text{CCI}_2$ and that of CO per mg of microsomal protein were compared, the microsomes from PB-treated rats showed an elevenfold higher carbene formation (0.39 nmol/mg protein) and a threefold greater CO production (0.74 nmol/mg protein) in comparison with microsomes from corn oil- or $\beta$-NF-treated animals (Table 1). When $\text{CCI}_2$ and CO production were expressed per nmol cytochrome P-450, again both $\text{CCI}_2$-P-450 and CO generation were at all time points significantly greater with microsomes from PB- and, to a lesser extent, Aroclor-treated rats than with microsomes from corn oil or $\beta$-NF-induced animals. These results indicate that the forms of cytochrome P-450 induced in the rat by PB or Aroclor metabolize $\text{CCI}_4$ more efficiently.

A different stability of the carbene-cytochrome P-450 complexes in liver microsomes from rats with different pretreatment was also found in the present work. Using the ratio between total carbene
complex formation (\(\text{CCI}_2\cdot\text{P}-450 + \text{CO-P}-450\)) and degradation (\(\text{CO-P}-450\)) as an index of the stability of the carbene complex, it was found that microsomes from PB- and Aroclor-treated rats were characterised by a higher stability of the complex than microsomes from corn oil- or \(\beta\)-NF-treated rats (Fig. 7). Wolf et al. (1977) reported that carbon monoxide formation in microsomes from 3,4-benzpyrene-induced rats was even higher than that observed with microsomes from PB-treated rats. It was concluded by these authors that the benzpyrene-induced form of cytochrome P-450 did not stabilize the carbene as well.

These findings overall indicate that the apoprotein of cytochrome P-450 plays a critical role in modulating the stability of the carbene complex. A possible reason for the different stability observed might be a higher affinity of some forms of cytochrome P-450 for the carbene ligand, which decreases its rate of dissociation from haem and the subsequent hydrolysis.

After discussing the evidence for carbene formation and hydrolysis to CO, the obvious point to be considered is the question whether carbene species are involved in the reductive inactivation of microsomal cytochrome P-450 by \(\text{CCI}_4\).

An important, potentially critical role of the carbene species in cytochrome P-450 inactivation and other aspects of \(\text{CCI}_4\) toxicity has already been proposed (Lange & Mansuy, 1981; Nastainczyk et al.,
It has been suggested that the haem moiety of cytochrome P-450 might be alkylated by :CCl₂ (de Groot & Haas, 1981) resulting in the inactivation of the haemoprotein. This hypothesis is supported by a number of studies in which the chemical properties and the potential biological implications of carbene species have been investigated. The formation of stable iron-porphyrin complexes upon reaction of CCl₄ with Fe(II)-tetraphenyl porphyrin in presence of a reducing agent has been known for some time in organometallic chemistry (Mansuy et al., 1980). These complexes have been successfully used to mimic the active site of cytochrome P-450 and to study or even to predict the properties of the corresponding cytochrome P-450-carbene complexes (Mansuy, 1980). A biphasic model system for both reductive and oxidative cytochrome P-450-dependent metabolism of CCl₄ has been described (Mansuy et al., 1980). In this system meso-tetraphenylporphyrin-Fe (Fe(TPP)) maintained in its ferrous state by reducing agent in excess was shown, under anaerobic conditions, to form FeII(TPP)-CCl₂ complexes and, in presence of oxygen, to convert CCl₄ to phosgene.

Two possible biological consequences of the formation of carbene-P-450 complexes have been considered (Mansuy, 1980). In one case, if the iron-carbon bond is very stable the sixth coordination position of the iron will be blocked and, under aerobic conditions, prevented from reacting with oxygen, thus resulting in the inhibition of the
monooxygenase function of the cytochrome. If more reactive carbene species are formed, these could be released from the haem iron and bind the prosthetic group or the apoprotein of cytochrome P-450 irreversibly. It has been speculated that the in vivo CCl₄-dependent degradation of cytochrome P-450 could be due to the formation of these cytochrome P-450-carbene complexes possibly resulting in the N-alkylation of the haem moiety and the formation of N-substituted porphyrins (Lange & Mansuy, 1981). A critical role of dichlorocarbene in the inactivation of cytochrome P-450 is not supported, however, by the results of the present work, since the carbene trapping agent DMB was unable to protect cytochrome P-450 haem against CCl₄-dependent destruction. These findings will be described and discussed in another section of the present thesis (see 3.3.1.5).

An attempt was made to modify the cofactors involved in the reductive activation of CCl₄ by the microsomal cytochrome P-450 system in order to see whether these factors had any effect on the formation of specific CCl₄ reactive metabolites and/or on the inactivation of the cytochrome by CCl₄. Preliminary experiments on the effect of FMN, the prosthetic group of NADPH-cytochrome P-450 reductase, on the CCl₄- and NADPH-dependent formation of the carbene complex were difficult to interpret. An enhancing effect of FMN on the rate of formation of the peak at about 460 nm was observed in
the NADPH-reduced difference spectrum, both in presence and in absence of Hb. Further work seemed to indicate, however, that the effect of FMN was only seen when NADPH, whether added or produced by a generating system, but not when the potent chemical reductant sodium dithionite was used to reduce microsomes. This observation and the fact that the absolute spectrum of oxidised FMN has a large peak at about 450 nm, suggested that the effect of FMN might be simply due to relatively higher amounts of oxidised FMN being present in test as compared to reference cuvette. It could be speculated that the substrate, present in test but not in reference cuvette, somehow modulated (by decreasing) the flow of electron equivalents provided by the reductase to exogenous FMN.

If this was the case, one has to conclude that, unlike with other types of substrates (e.g. azo dyes) FMN did not increase the rate of reduction of CCl₄ by cytochrome P-450. Consistent with this interpretation are also i) the lack of effect of FMN on CO production observed when Hb was present in the incubation mixture and ii) the only partial inhibition of the carbene peak by the carbene-trapping agent DMB observed in presence of FMN.

An interesting observation was the formation of a reduced difference spectrum of haem, as methaemalbumin, on addition of CCl₄ to anaerobic, microsome-free, sodium dithionite-reduced incubations (Fig. 8). The peak at about 418 nm formed in this spectrum on
addition (or in presence) of Hb to the non-enzymic incubation (Fig. 9) provided evidence for the formation of CO in a similar manner to that occurring with cytochrome P-450. This indicated that under reducing conditions non-protein haem can interact with CCl₄ in a way which seems to mimic the interaction of the substrate with the active site of cytochrome P-450. Based on this assumption it was postulated that other suicide substrates of cytochrome P-450 could also interact with haem in a similar way. This in fact was found to be the case with the anaesthetic halothane, (see 4.3). Based on these consistent results obtained with CCl₄ and halothane, a general mechanism of non-enzymatic reductive activation of polyhalogenated chemicals by haem compounds will be proposed. This mechanism has been investigated in some detail and found to require only: (i) the suicide substrate, (ii) a ligand-free haem iron and (iii) electron donation.

In conclusion, the spectral changes induced in anaerobic liver microsomes by CCl₄ can be used, to some extent, to investigate the reductive metabolites of CCl₄ and their interactions with the prosthetic haem of cytochrome P-450. Moreover, CCl₄-dependent spectral changes were also seen with haem alone under anaerobic conditions. This suggests a potential for reduced haem to interact with and activate CCl₄ even in the absence of the apoprotein.
3. **Carbon tetrachloride-dependent cytochrome P-450 inactivation.**

3.1 **INTRODUCTION**

It is now well known that during anaerobic metabolism of CCl₄ by NADPH-reduced liver microsomes, cytochrome P-450 is rapidly inactivated (Yamazoe *et al.*, 1979) and protohaem, the prosthetic group of the cytochrome, is also lost (de Groot & Haas, 1980 and 1981). It has been proposed that reactive metabolites such as the trichloromethyl radical (.CCl₃) or dichlorocarbene (:CCl₂) formed during metabolic activation of CCl₄ may attack and irreversibly modify the haem moiety of cytochrome P-450 (Fernandez *et al.*, 1982; Guzelian & Swisher, 1979). This is in agreement with the observation that the forms of cytochrome P-450 which are known to activate CCl₄ to reactive species - such as those induced in the rat by pretreatment with PB or ethanol - are also primarily susceptible to destruction by CCl₄ (Noguchi *et al.*, 1982a; Gadeholt, 1984). However, the reactive metabolite of CCl₄ directly responsible for microsomal haem and cytochrome P-450 destruction, and the molecular mechanism of the process are not known.

Evidence is presented that haem is the primary, suicidal target of the reductive activation of CCl₄ by cytochrome P-450. It will be shown that both enzymatic and non-enzymatic mechanisms of CCl₄ activation may result in suicidal haem loss, and that the loss of
haem is due to irreversible modification of its porphyrin structure. Moreover, using the selective carbene-trapping agent DMB, the role of \( \text{CCL}_2 \) on \( \text{CCL}_4 \)-dependent cytochrome P-450 haem destruction is investigated in liver microsomes from PB-treated rats.

3.2. MATERIALS AND METHODS

3.2.1 Chemicals and biochemicals

Protoporphyrin IX dimethyl ester was purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Mesoporphyrin IX and deuteroporphyrin IX were obtained from Koch-Light Ltd. All these porphyrins were obtained as the methyl esters and these were then hydrolysed to the free acids as described in 3.2.4.3.

Methaemalbumin was prepared as described in 2.2.1. An identical procedure was used to prepare a protoporphyrin IX-human albumin complex, and mesohaem- or deuterohaem-human albumin complexes using unlabelled mesohaem or deuterohaem. These two haem analogues, obtained by inserting iron into the corresponding porphyrin carboxylate (mesoporphyrin IX and deuteroporphyrin IX, respectively), were a gift of Dr. F. De Matteis (MRC Toxicology Unit, Carshalton, Surrey, U.K.).

NADPH-cytochrome P-450 reductase (EC 1.6.2.4), purified from hamster liver by the method of Yasukochi & Masters (1976), was a gift of Dr. C. Reed (MRC Toxicology Unit, Carshalton, Surrey).
The other chemicals were purchased, or prepared as described in 2.2.1., and were of analytical grade.

3.2.2 Haem assay by the pyridine/haemochrome reaction

Microsomal and methaemalbumin haem were measured using the method of Paul et al. (1953) by either the reduced absolute spectrum or the reduced minus oxidised difference spectrum in pyridine/NaOH. In the case of the reduced absolute spectrum the usual assay procedure was the following. Before each incubation, a baseline was recorded between 570 and 530 nm with a freshly prepared pyridine/0.3 M NaOH mixture (15:27, v:v) in both test and reference cuvettes. At the end of each incubation time, 0.8-0.9 ml of incubation mixture were pipetted into a 3-ml glass cuvette containing 1.87-2.1 ml of the pyridine/NaOH mixture and stoppered by a teflon cap. Then a few mg solid sodium dithionite were added to the mixture, which was mixed gently and after 1 or 2 min, when haem was maximally reduced, a difference spectrum between 570 and 530 nm was recorded against pyridine/NaOH. This method was preferentially used when the haem was known or was expected to be already reduced at the end of the incubation.

When oxidised haem had to be measured the alternative method was used instead (for instance to determine the concentration of freshly prepared methaemalbumin). In this case microlitre amounts of the
concentrated methaemalbumin solution were added to 6 ml of the pyridine/NaOH mixture. After mixing, 2.5 ml amounts of this new solution were pipetted into two 3-ml glass cuvettes. Then a few mg sodium dithionite were added to the test cuvette and 50 ul of 3 mM K$_3$Fe(CN)$_6$ were pipetted into the reference cuvette, before recording, after 2-3 min, a difference spectrum between 570 and 530 nm in a dual beam UV-VIS spectrophotometer model Varian, Cary 219 or 2200. Sodium dithionite and potassium ferricyanide have no absorbance at these wavelengths. An εM$^{-1}$.cm$^{-1}$ 557-541 20.7 (Falk, 1964a) was used for both types of spectra to calculate haem concentration. From the values obtained, unless otherwise indicated, the contribution of catalase haem present in the oxygen-scavenging system was finally subtracted as it was known from preliminary experiments that catalase haem does not undergo CCl$_4$-dependent inactivation.

This method could not be used to measure the loss of haem during cytochrome P-450 inhibition studies where CO is bubbled through the incubation mixture, since it was found that CO prevents the interaction of pyridine with the haem iron as described below.
3.2.2.1 Carbon monoxide inhibition of the pyridine/haemochrome reaction

The inhibitory effect of CO on the reduced pyridine-haemochrome spectrum was investigated as follows. Haem (5.5 nmoles as MHA in 0.8 ml 0.1 M Na₂HPO₄ aerobic buffer, pH 7.4) was added to 1.87 ml pyridine/0.3 M NaOH (15:27, v:v) in each of two 3-ml rubber-stoppered glass cuvettes. The mixture was mixed gently and a baseline was recorded between 600 and 500 nm (Fig. 10).

Then a few mg solid sodium dithionite or K₃Fe(CN)₆ were added to test and reference cuvettes, respectively, and the reduced minus oxidised spectrum was recorded showing typical α and β peaks at 556 and 525 nm, respectively. On titration with CO-saturated buffer the 556 nm peak decreased progressively and became a trough at about 50 uM CO (corresponding to "+ 146 ul of 1 mM CO" in the Figure), while an isosbestic point was observed at 565/6 nm. This suggested that a bathochromic shift of the α peak had occurred. When the solution of the test cuvette was finally saturated with CO, only two new peaks, one at 560 nm and the other at 538 nm, and a trough at 556 nm were present in the spectrum (Fig. 10).
Fig. 10. Effect of CO on the difference (reduced minus oxidised) pyridine/haemochrome spectrum. After recording a reduced minus oxidised difference spectrum of haem in pyridine/0.3 M NaOH (15:27, v:v) ("before CO" in the Figure) microlitre amounts (1 to 146 µl) of a CO-saturated water solution were added to the test cuvette and scans were recorded after each addition. Finally, the test cuvette was saturated with CO before recording the last spectrum (indicated by "1 mM CO" in the Figure). The reference cuvette contained an equal concentration of ferricyanide-oxidised haem throughout.
3.2.3 Concurrent protohaem and mesohaem determination

A simple and rapid spectrophotometric method was developed to measure concurrently protohaem and its non physiological analogue mesohaem in the same incubation. This method was used to distinguish the loss of microsomal haem from that of exogenous haem during the reductive activation of $\text{CCl}_4$ by liver microsomes to which exogenous mesohaem had been added. The method is a modification of that proposed by Porra (1976) for measuring the activity of ferrochelatase (EC 4.99.1.1) in presence of contaminating haemoglobin. The present modified method, however, has the advantage that quantitation of both protohaem and mesohaem is possible in the presence of variable concentrations of both.

The assay involves the recording of the absolute dithionite-reduced spectrum between 600 and 500 nm of the mixture in pyridine/NaOH. The concentration of protohaem and mesohaem are then obtained by simple algebraic calculations from the values of $\Delta A_{556-539}$ and $\Delta A_{546-530}$ measured in this spectrum using the formulae below (modified from Porra (1976)) and the following extinction coefficients calculated from the absolute dithionite-reduced spectra in pyridine/NaOH of standard solutions of proto- and mesohaem. The haem concentration in these standard solutions was calculated using the classical extinction coefficients for the reduced minus oxidised
spectra in pyridine/NaOH reported by Falk (1964a), i.e. $\varepsilon_{\text{mM}^{-1} \cdot \text{cm}^{-1}}$

20.7 for protohaem and $\varepsilon_{\text{mM}^{-1} \cdot \text{cm}^{-1}}$ 547-531 21.7 for mesohaem.

The values obtained for the absolute spectra were $\varepsilon_{\text{mM}^{-1} \cdot \text{cm}^{-1}}$

20.9 and $\varepsilon_{\text{mM}^{-1} \cdot \text{cm}^{-1}}$ 546-530 0.0 for protohaem, and $\varepsilon_{\text{mM}^{-1} \cdot \text{cm}^{-1}}$ 539-556 7.5 and $\varepsilon_{\text{mM}^{-1} \cdot \text{cm}^{-1}}$ 546-530 20.0 for mesohaem. These values were similar but not identical to those reported by Porra (1976).

\[
(5) \quad M = \frac{d (a_1 - b_2)}{p_1 m_2 + p_2 m_1} \\
(6) \quad P = \frac{d (a_2 + b_1)}{p_2 m_2 + p_1 m_1}
\]

where:

$M$ = concentration of mesohaem (mM)
$P$ = concentration of protohaem (mM)
\(d\) = dilution factor
\(a = \Delta A_{546-530}\)
\(b = \Delta A_{556-556}\)

\(p_1 = \varepsilon_{\text{mM}^{-1} \cdot \text{cm}^{-1}} 556-539\) for protohaem
\(p_2 = \varepsilon_{\text{mM}^{-1} \cdot \text{cm}^{-1}} 546-530\) for protohaem
\(m_1 = \varepsilon_{\text{mM}^{-1} \cdot \text{cm}^{-1}} 546-530\) for mesohaem
\(m_2 = \varepsilon_{\text{mM}^{-1} \cdot \text{cm}^{-1}} 539-556\) for mesohaem

In order to test the method, 5 different mixtures containing known concentrations of proto- and mesohaem were prepared and their absolute dithionite-reduced spectra in pyridine/NaOH were recorded.

From these spectra (Fig. 11) the concentrations of the two components were calculated using the formulae (5) and (6) above.
The comparison between expected and calculated concentrations of protohaem and mesohaem is reported in Table 2.
Fig. 11. Absolute dithionite-reduced alkaline-pyridine spectra of five mixtures of protohaem and mesohaem. The mixture of spectrum 1 contained only mesohaem and that of spectrum 5 contained only protohaem, whereas the mixtures of spectra 2 to 4 contained different ratios of the two components as indicated in Table 2.
Table 2. Accuracy of the spectrophotometric method for the concurrent measurement of proto- and mesohaem.

<table>
<thead>
<tr>
<th>Mixture No.</th>
<th>Proto (µl)</th>
<th>Meso (µl)</th>
<th>Proto accuracy (µM) (%)</th>
<th>Meso accuracy (µM) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0</td>
<td>10.0</td>
<td>0.02 (0.00) -</td>
<td>6.98 (7.00) 99</td>
</tr>
<tr>
<td>2</td>
<td>2.5</td>
<td>7.5</td>
<td>1.68 (1.79) 94</td>
<td>5.30 (5.25) 99</td>
</tr>
<tr>
<td>3</td>
<td>5.0</td>
<td>5.0</td>
<td>3.48 (3.58) 97</td>
<td>3.57 (3.50) 98</td>
</tr>
<tr>
<td>4</td>
<td>7.5</td>
<td>2.5</td>
<td>5.46 (5.37) 98</td>
<td>1.81 (1.75) 97</td>
</tr>
<tr>
<td>5</td>
<td>10.0</td>
<td>0.0</td>
<td>7.15 (7.17) 99</td>
<td>0.02 (0.00) -</td>
</tr>
</tbody>
</table>

The indicated increasing volumes of stock protohaem solution (Proto, 2.15 mM, as methaemalbumin) were added to pyridine/NaOH together with decreasing volumes of stock mesohaem solution (Meso, 2.10 mM, as methaemalbumin) in five different ratios, before recording the absolute reduced spectrum between 600 and 500 nm.
Fig. 12. Correlation between calculated and expected concentrations of proto- and mesohaem. Values are those reported in Table 2.
An accuracy $\geq 94\%$ was obtained with all ratios tested. When the correlation between calculated and expected concentrations was calculated, a good correlation coefficient ($r > 0.99$) was found for both haems (Fig. 12).

3.2.4 Protoporphyrin assay

Two methods were used for the measurement of protoporphyrin IX: the oxalic acid method and the perchloric acid method.

3.2.4.1 Oxalic acid method (Morrison, 1965)

Solid oxalic acid was washed with two volumes (w:v) of twice distilled water at 4°C. A saturated solution of oxalic acid was then prepared in order to obtain an approximately 2 M stock solution. One or two hundred ul of incubation mixture were added to 1.8-1.9 ml saturated oxalic acid solution in a 15-cm glass tube with a glass ball cap. The mixture was mixed and immediately heated for 30 min at 98-100°C by inserting the lower half of the tube in a rack which was placed in a boiling water bath. In this way evaporation from the tube was minimized. After cooling, the water vapour condensed onto the glass was recovered from the tube walls and the cap. After mixing, 1 ml of the solution was pipetted into a 1 ml glass microcell which was inserted into a microcell holder and the fluorescence was read in a luminescence spectrometer model Perkin
Elmer LS-5 using 404 and 603 nm as the excitation and emission wavelengths, respectively. The concentration of protoporphyrin IX was then calculated using calibration curves obtained, as described in 3.2.4.4, with standard solutions of authentic protoporphyrin IX prepared as described in 3.2.4.3. These wavelengths were selected from excitation and emission spectra of appropriate authentic protoporphyrin IX solutions.

3.2.4.2 Perchloric acid method (Grandchamp et al., 1980)

This fluorescence technique is milder than the oxalic acid method and allows the measurement of preformed porphyrins even in the presence of haem compounds in the incubation mixture. It can therefore be used, for example, to assay mixtures containing significant amounts of contaminating haemoglobin or other haemoproteins, or when the concurrent determination of porphyrin and haem is required. The procedure is the following. At the end of the incubation, 0.3 ml incubation mixture was pipetted into 3 ml methanol/1N-HClO₄ (1:1, v:v) and the fluorescence was determined in a luminescence spectrometer model Perkin Elmer LS-5 using 404/603 nm as the excitation and emission wavelengths, respectively. A calibration curve was prepared, as described in 3.2.4.4, using standard solutions of authentic protoporphyrin IX dimethyl ester which was hydrolysed, dried and dissolved in methanol/1N-HClO₄.
before fluorescence measurement.

3.2.4.3 Hydrolysis of protoporphyrin IX dimethyl ester (PDE):

Protoporphyrin IX free acid was obtained by hydrolysis of PDE as follows. A few mg solid PDE were dissolved in 10 ml dried chloroform and the concentration of PDE in this solution was measured in a teflon-stoppered glass cuvette by its absolute aetio-type spectrum using the $\varepsilon_{407 \text{ M}^{-1} \cdot \text{cm}^{-1}} = 171$ reported by Falk (1964a). The solution was diluted with more chloroform to obtain a 1 uM stock solution to be used for the calibration curve as described below and transferred to conical glass test tubes. Chloroform was then evaporated by gentle flushing with nitrogen before addition of 0.3 ml 6 N HCl to redissolve PDE. After gentle mixing the tubes were stored at room temperature in the dark for 4-5 hours. After hydrolysis the HCl was evaporated under nitrogen until a negative pH paper test was obtained. One tube to which 1000 pmol PDE were originally added was used to measure the recovery of protoporphyrin IX after hydrolysis. Five ml of 1.37 N HCl were added to this tube to dissolve the porphyrin and, after gentle mixing, 3 ml of the solution were transferred to a glass cuvette and the concentration of hydrolysed protoporphyrin was measured by its absolute spectrum using $\varepsilon_{408 \text{ M}^{-1} \cdot \text{cm}^{-1}} = 262$ (Falk, 1964a). From this value the total amount of protoporphyrin IX in the tube was calculated (90 to 95% of
protoporphyrin IX added initially was usually recovered after hydrolysis).

3.2.4.4 Calibration curve of protoporphyrin IX

To obtain a calibration curve increasing amounts (0 to 1000 ul) of a 1uM stock solution of PDE in chloroform were pipetted in triplicate in the conical bottom of 10-ml glass test tubes and taken to a volume of 1 ml with chloroform. After evaporation of chloroform and hydrolysis of PDE as described above, approximately 100 ul of a 1% human albumin solution in Tris/NaOH mixture (48 mg Tris in 10 ml 0.1 N NaOH, pH adjusted to 7.4 with 1 N HCl) were added to dissolve protoporphyrin IX. After mixing, 1.9 ml saturated oxalic acid were pipetted into each tube and the mixture was transferred into a 3-ml quartz cuvette or 1-ml glass minitubes. In the latter case a microcell adaptor had to be used to fix the minitube to the instrument cell holder. Finally, the fluorescence was measured using 409/606 as the excitation/emission wavelengths in a Perkin Elmer model LS-5 luminescence spectrometer. A good correlation (r > 0.99) with a linear distribution over the all range of concentrations tested was usually obtained between protoporphyrin IX concentration and blank-corrected fluorescence values (data not shown). No significant increase in fluorescence was noted after heating the porphyrin/oxalic acid mixture for 30 min at 98-100°C.
When the loss of haem-derived protoporphyrin IX had to be studied, a double calibration was performed using standard solutions containing equimolar amounts of authentic protoporphyrin IX and protohaem (both as the human albumin complexes prepared as described in 2.2.1). The two standard curves were superimposable as authentic and haem-derived protoporphyrin IX gave very similar fluorescence values to each other (Fig. 13).

Pretreatment of animals, preparation of microsomes, determination of cytochrome P-450, cytochrome b₅ and microsomal or mitochondrial protein, general procedure of anaerobic incubations and oxygen-scavenging system were as described in 2.2.
Fig. 13. Calibration curve of authentic protoporphyrin IX and haem-derived protoporphyrin IX. Standard solutions were prepared by adding equal amounts (3.1, 6.2, 9.3 and 12.4 nmoles) of haem (as MHA) or protoporphyrin IX (as protoporphyrin IX free acid-human albumin complex) to 2.5 ml Na₂HPO₄ buffer, pH 7.4, containing the usual oxygen-scavenging system. After mixing, all solutions were heated at 98-100°C for 30 min. After heating, 110 μl of each solution were pipetted into 2.09 ml saturated oxalic acid solution and mixed before measuring the fluorescence as described in 3.2.4.1.
3.3 RESULTS

3.3.1 CCl₄-dependent loss of microsomal cytochrome P-450

When NADPH-reduced liver microsomes were incubated under anaerobic conditions (as described in 2.2.7) in presence of CCl₄, a parallel loss of cytochrome P-450 and haem was observed. No loss of cytochrome b₅ was observed in these experiments (results not shown). Moreover, in a second group of experiments it was found that the loss of haem was accompanied by equimolar loss of protoporphyrin IX and was prevented by CO. These observations are now described.

3.3.1.1 Destruction of microsomal cytochrome P-450 involves parallel destruction of haem prosthetic group

When, in two separate, identical experiments, NADPH-reduced liver microsomes were incubated anaerobically with CCl₄, an approximately 60% loss of total cytochrome P-450 initially present was observed over a period of 5 min (Fig. 14).

The loss of haem, as measured by the pyridine/haemochrom reaction, was parallel and equimolar to that of cytochrome P-450 when monitored over a period of up to 30 min. When CCl₄ or NADPH was omitted from the incubation the loss of both cytochrome P-450 and haem was negligible over the period of 30 minutes. The higher concentration of haem as compared to that of cytochrome P-450
Fig. 14. Equimolar loss of cytochrome P-450 and haem during anaerobic incubation of NADPH-reduced rat liver microsomes with CCl₄. Liver microsomal protein (0.74 mg/ml) from PB-treated rats containing 1.83 ± 0.11 nmol cytochrome P-450/mg was incubated in rubber-stoppered glass cuvettes at 37°C in 2.5 ml anaerobic buffer, containing a NADPH-generating system comprising G-6-PD (0.15 U/ml), G-6-P (1.9 mM) and NADP⁺ (0.12 mM). At time 0 2.7 umol CCl₄ in methanol were added to start the reaction. The reaction was stopped either by injecting into the incubation mixture 100 µl of a CO-saturated solution of sodium dithionite in anaerobic buffer for cytochrome P-450 determination, or by pipetting 0.9 ml of incubation mixture into 2.1 ml pyridine/NaOH for haem measurement. Cytochrome P-450 was measured by the difference in absorbance between 450 and 490 nm in the reduced, CO/absolute spectrum as described in 2.2.3. Values are the average of 2 experiments.
measured at all incubation times was due to haem from cytochrome b_{5} which in preliminary experiments did not undergo CCl_{4}-dependent inactivation.

When sodium dithionite was used as the 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 P-450 was accompanied by an equimolar loss of microsomal haem (data not shown).

3.3.1.2 The loss of haem involves irreversible modification of the tetrapyrrolic structure and is inhibited by carbon monoxide

In order to exclude that the loss of the pyridine haemochrome reaction might be due to masking of the haem iron by some CCl_{4} metabolites, making it inaccessible to the pyridine, the haem was also measured by conversion to protoporphyrin IX and determination of the resulting fluorescence in oxalic acid (see 3.2.4.1).

In this experiment, in which the haem concentration was measured in the same samples using the two methods, an equimolar loss of the pyridine/haemochrome reaction and porphyrin fluorescence was found on addition of CCl_{4} to dithionite-reduced liver microsomes (Table 3). This is consistent with the hypothesis that on CCl_{4} attack haem undergoes a structural change of its tetrapyrrolic macrocycle. In the same experiment and using the fluorimetric technique it was also
Table 3. CCl₄-dependent loss of haem as measured by either the pyridine/haemochrome reaction or the porphyrin fluorescence assay. Effect of carbon monoxide.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Pyridine/haemochrome</th>
<th>Porphyrin IX</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>3.45±0.20 (100%)</td>
<td>3.35±0.23 (100%)</td>
</tr>
<tr>
<td>Sodium dithionite (D)</td>
<td>3.68±0.20 (106%) NS</td>
<td>3.15±0.30 (94%) NS</td>
</tr>
<tr>
<td>D and CCl₄</td>
<td>2.15±0.16 (62%) **</td>
<td>2.06±0.19 (61%) *</td>
</tr>
<tr>
<td>D, CCl₄ and CO</td>
<td>£</td>
<td>2.96±0.15 (88%) NS</td>
</tr>
</tbody>
</table>

NS, not significant; *, p < 0.01; **, p < 0.001, when compared to the control values (Student's t test). £, not measurable (see 3.2.2.1). One mg of liver microsomal protein (2.60 ± 0.01 nmol cytochrome P-450/mg) from PB-treated rats was incubated at 23°C in 1 ml of the usual anaerobic buffer with 14.3 mM sodium dithionite. At time 0, CCl₄ was injected to obtain a final concentration of 1 mM. In one incubation CO was bubbled through the microsomal suspension for 30 sec before addition of dithionite. After a 10 min incubation 100 ul portions of the incubation mixture were pipetted into 1.9 ml saturated oxalic acid for porphyrin assay and 0.7 ml portions to 1.63 ml of pyridine/NaOH for pyridine/haemochrome assay. Values are mean ± SD of three determinations. The percentage of the corresponding control value is given in parentheses.
possible to test the inhibitory effect on the haem loss of saturating concentrations of exogenous CO. This effect could not be studied using the pyridine/haemochrome method since, as already mentioned, CO interferes with this method in that it apparently prevented the interaction of pyridine with the reduced haem iron (see 3.2.2.1).

3.3.1.3 High, but not low carbon monoxide concentrations inhibit the \( \text{CCl}_4 \)-dependent loss of cytochrome P-450

The protective effect of CO on the \( \text{CCl}_4 \)-dependent inactivation of cytochrome P-450 was investigated further by incubating liver microsomes from PB-treated rats with different concentrations of \( \text{CCl}_4 \) and CO in presence of sodium dithionite (Fig. 15).

In this experiment the classical peak at 450 nm due to the CO-cytochrome P-450 complex was used to quantitate the haemoprotein as described in 2.2.3.

After a 10 min incubation of microsomes with saturating CO (about 1 mM) at 20°C, the decrease of the CO-cytochrome P-450 spectrum, as compared to the initial value, was 1.4% with dithionite alone and 4.6% with dithionite plus \( \text{CCl}_4 \) (Fig. 15). On the contrary, a 44% \( \text{CCl}_4 \)-dependent decrease of the cytochrome P-450 peak was measured after 10 min (reaching a 53% loss after 60 minutes) in the incubation containing 50 \( \mu \text{M} \) CO. Note that this concentration of CO
Fig. 15. Effect of CO on the CCl₄-dependent inactivation of cytochrome P-450. Equal amounts of microsomal protein (0.95 mg, 2.60 ± 0.01 nmol cytochrome P-450/mg) were preincubated at 20°C for 5 min in 1 ml of the usual anaerobic buffer in each of two 1 ml plastic cuvettes. After recording a baseline between 530 and 400 nm, CO was bubbled through the test cuvette (1 bubble/sec for 1 min) and approximately 2.5 mg sodium dithionite in solution were added to both cuvettes. At time 0, in order to start the reaction, CCl₄ in methanol was injected into the test cuvette to obtain a final concentration in the incubation mixture of 1 mM. A control incubation with dithionite but without CCl₄ addition was also performed. In another incubation CO was bubbled through the dithionite solution before injection of the latter into the test cuvette. This gave a CO concentration in the incubation mixture of about 50 μM. In all cases scans were recorded at 1, 3, 5, 10, 20, 30, 40, 50 and 60 minutes after the addition of CCl₄. The difference in absorbance between 450 and 490 nm was used to measure cytochrome P₄₅₀ concentration.
is approximately 20 fold higher than that of the initial cytochrome P-450 present but 20 fold lower than that of CCl\textsubscript{4}.

With the same microsomal preparation about the same loss of the initial haemoprotein was known from previous experiments to occur under these conditions in the absence of CO (Table 3). These data indicate that protection by CO against the CCl\textsubscript{4}-dependent loss of cytochrome P-450 in dithionite-reduced microsomes was effective when high (saturated) but not low concentrations of CO were used.

3.3.1.4 Effect of rat pretreatment on CCl\textsubscript{4}-dependent cytochrome P-450 inactivation

The effect of various inducers of cytochrome P-450 on the suicidal inactivation of the haemoprotein by CCl\textsubscript{4} was investigated and the results are reported in Table 4.
Table 4. Effect of rat pretreatment on the CCl₄-dependent loss of cytochrome P-450 haem from NADPH-reduced liver microsomes.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Microsomal haem (nmol/mg protein)</th>
<th>Loss of haem (nmol/mg) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>initial</td>
<td>final</td>
</tr>
<tr>
<td>Corn oil</td>
<td>1.48 ± 0.04</td>
<td>1.01 ± 0.07</td>
</tr>
<tr>
<td>PB</td>
<td>2.35 ± 0.10</td>
<td>1.31 ± 0.01</td>
</tr>
<tr>
<td>Aroclor</td>
<td>2.92 ± 0.13</td>
<td>1.37 ± 0.07</td>
</tr>
<tr>
<td>β-NF</td>
<td>1.76 ± 0.02</td>
<td>1.34 ± 0.04</td>
</tr>
</tbody>
</table>

Microsomes (1 mg protein/ml) from rats treated with corn oil, PB, Aroclor and β-NF (containing 0.77, 1.62, 2.4 and 1.17 nmol cytochrome P-450/mg, respectively) were incubated at 25°C for 10 min with NADPH (1 mM) and CCl₄ (1 mM) in the usual anaerobic buffer. At the end of the incubation 0.8 ml were pipetted into pyridine/NaOH for haem assay. Values are mean ± SD from at least 3 determinations.
Aroclor and PB pretreatment stimulated the haem loss while \( \beta \)-NF pretreatment decreased the haem loss when compared to the control treatment (corn oil). These results resemble those obtained when the effects of rat pretreatment on the formation of \( \text{CCl}_2 \) and CO were investigated and indicate that \( \text{CCl}_4 \) metabolism and cytochrome P-450 inactivation are closely related.

3.3.1.5 Effect of the carbene trapping agent DMB on the \( \text{CCl}_4 \)-dependent cytochrome P-450 destruction

In order to investigate whether \( \text{CCl}_2 \) contributes as a reactive species to the destruction of the haemoprotein, the specific carbene-trapping agent DMB was included in the incubation mixture and the loss of microsomal haem was investigated during the anaerobic incubation of NADPH- or sodium dithionite-reduced microsomes with \( \text{CCl}_4 \). DMB did not significantly prevent the \( \text{CCl}_4 \)-dependent loss of haem in NADPH-reduced incubations (Table 5) nor in dithionite-reduced incubations where a 25% and 22% loss of haem was found in the absence and presence of DMB, respectively (Fig. 6).

3.3.2 The \( \text{CCl}_4 \)-dependent loss of cytochrome P-450 haem is a typical suicide inactivation reaction

The results reported above (see 3.3.1.1 and 3.3.1.2) are in agreement with the suggestion that haem is both the site and the
Table 5. Lack of effect of DMB on the CCl₄-dependent loss of haem with reduced liver microsomes from PB-treated rats

<table>
<thead>
<tr>
<th>Addition</th>
<th>Haem (nmol/mg prot.)</th>
<th>Haem loss (nmol/mg (% of prot.) control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>2.63 ± 0.04</td>
<td>-</td>
</tr>
<tr>
<td>NADPH</td>
<td>2.47 ± 0.03 *</td>
<td>0.16</td>
</tr>
<tr>
<td>NADPH and CCl₄</td>
<td>1.62 ± 0.03 **</td>
<td>1.01</td>
</tr>
<tr>
<td>NADPH, CCl₄ and DMB</td>
<td>1.69 ± 0.02 ** &amp;</td>
<td>0.94</td>
</tr>
</tbody>
</table>

*, p < 0.01, when compared with control value; **, p < 0.001, when compared with value obtained with NADPH alone; &, not significant, when compared with value obtained with NADPH and CCl₄ (Student's t test). Rat liver microsomal protein (1.9 mg) containing 1.95 nmol cytochrome P-450/mg was incubated for 10 min at 23°C in 2 ml anaerobic buffer. Where indicated the following additions were made (final concentration in parentheses): NADPH (1 mM), CCl₄ (1 mM) and DMB (5 mM). At the end of the incubation 0.8 ml of incubation mixture were transferred to pyridine/NaOH for haem assay. Values are mean ± SD of 3 determinations.
target of CCl₄ activation by cytochrome P-450. These are conditions necessary but not sufficient to consider cytochrome P-450 loss a classical suicide inactivation reaction according to the established definition of this process in enzymology. Further evidence to support this hypothesis is described now and elsewhere in the present thesis (see 5.3.1).

3.3.2.1 Kinetics of CCl₄-dependent cytochrome P-450 inactivation

As shown in 3.3.1.1, and in other preliminary experiments not reported, in which liver microsomes from Aroclor-treated rats were used, a maximum 60% loss of cytochrome P-450 was achieved after a 15-20 min incubation at 22°C of microsomes containing micromolar concentrations of this haemoprotein with millimolar concentrations of CCl₄ and NADPH. This indicated that not all the haemoprotein was susceptible to CCl₄-dependent inactivation even in the presence of a three orders of magnitude higher concentration of substrate. Further evidence that the loss of cytochrome P-450 is a saturable process was obtained by incubating rat liver microsomes with CCl₄ and NADPH for various periods of time and measuring the loss of haem occurring during incubation. The results of this experiment are reported in Fig. 16.
Fig. 16. Inactivation kinetics of microsomal cytochrome P-450 by CCl$_4$. Liver microsomal protein from Aroclor-treated rats (1 mg/ml, 2.4 nmol cytochrome P-450/mg) was incubated at 22°C in anaerobic buffer in presence of 1 mM NADPH. At time 0, 60 nmol CCl$_4$ was added and after 30 sec and 1, 2.5, 5, 10 and 30 min the incubation was terminated by pipetting 0.8 ml in pyridine/NaOH for haem determination. The log % of the CCl$_4$-destructable haemoprotein initially present (1.45 nmol/mg protein) was plotted against incubation time after the addition of CCl$_4$. 
The loss of cytochrome P-450 haem showed pseudo first-order biphasic kinetics with calculated half-times of approximately 3.2 and 28.9 min. Decrease of microsomal haem indicates cytochrome P-450 loss, since under anaerobic conditions cytochrome b$_5$, the other haemoprotein present in significant amount in microsomes, is not vulnerable to CCl$_4$.

3.3.2.2 Partition ratio between catalytic activity and "suicidal" inactivation of cytochrome P-450

When concentrations of CCl$_4$ two orders of magnitude lower than those needed to achieve a maximum loss were used, cytochrome P-450 was still rapidly inactivated but the final loss was reached earlier and it was significantly smaller, indicating that limiting amounts of CCl$_4$ had been used. Based on this assumption the addition of further CCl$_4$ to the incubation mixture but not that of further NADPH would be expected to promote an additional loss of the haemoprotein. Experimental evidence showing that this was indeed the case is reported in Fig. 17.

In this experiment a maximal loss of haem (about 0.68 nmol/ml, corresponding to approximately 45-50% of the total inactivatable enzyme) was achieved after only 10-15 min of incubation in presence of CCl$_4$/NADPH. This was likely to indicate that at this point in time no further loss of haem was observed because no more substrate
Fig. 17. Haem loss during metabolism of limiting concentrations of 
CCl₄ by microsomal cytochrome P-450. Experimental conditions and 
components were as for Fig. 16 but for the initial concentration of 
CCl₄ in the incubation mixture (15 μM) and the measurement of 
microsomal haem at various times up to 40 min. In 3 other parallel, 
initially identical incubations, indicated by "+CCl₄", "+CCl₄/NADPH" 
and "+NADPH", additional CCl₄ and/or NADPH (2 umoles for both) were 
injected, respectively, at time 30 min in the incubation mixture and 
the haem concentration was measured at time 40 min. A 40-min 
control incubation containing 1 mM NADPH but no CCl₄ was also 
performed in triplicate.
was available for the reaction. This interpretation is supported by the following additional observations. The total amount of cytochrome P-450 susceptible to CCl₄-dependent destruction present in the incubation mixture between 15 and 30 min of incubation was not limiting, as 50-55% of the "suicide-prone" enzyme was known, from previous experiments, to be still undamaged. NADPH was also not limiting as the addition of more NADPH at 30 min did not increase the loss of enzyme. In contrast, the addition of more CCl₄ (2 umol) at 30 min, either alone or together with NADPH, was responsible for a further significant (approx. 40%) loss of inactivatable enzyme (Fig. 17). These findings overall support the previous interpretation that all the substrate initially present had been metabolized within 15 min.

So, based on this assumption, the partition ratio between metabolic and suicidal events (Wales, 1980), which indicates how often during metabolic turnover of CCl₄ by cytochrome P-450 will the catalytic cycle result in haem destruction, could be calculated. By using two different concentrations of substrate, very similar values of partition ratio were obtained, as reported in Table 6. The results indicate that approximately 26/27 molecules of CCl₄ were metabolised on average for every molecule of cytochrome P-450 inactivated.
Table 6. Partition ratio between metabolic turnover of CCl\textsubscript{4} and haem inactivation during suicidal activation of CCl\textsubscript{4} by microsomal cytochrome P-450.

<table>
<thead>
<tr>
<th>CCl\textsubscript{4} (nmol/ml)</th>
<th>Haem (nmol/ml)</th>
<th>Partition ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>initial *</td>
<td>final &amp; lost $</td>
</tr>
<tr>
<td>0</td>
<td>3.05</td>
<td>2.95</td>
</tr>
<tr>
<td>10</td>
<td>3.05</td>
<td>2.56</td>
</tr>
<tr>
<td>15</td>
<td>3.05</td>
<td>2.39</td>
</tr>
</tbody>
</table>

* Total haem concentration was actually 3.05 ±0.03 (mean ± SD) nmol/ml, including 2.4 nmol cytochrome P-450 haem/ml. &, Values indicate microsomal haem present at 20 min incubation when all CCl\textsubscript{4} has been metabolised. $, Values indicate CCl\textsubscript{4}-dependent loss of cytochrome P-450 haem after correction for the haem loss due to NADPH alone at 20 min (0.10 nmol/ml). Experimental conditions and components were as for Fig. 17 but for the indicated concentrations of CCl\textsubscript{4}. The reaction was terminated 20 min after the addition of CCl\textsubscript{4} when residual haem in the incubation mixture was determined by the pyridine/haemochromes reaction.
3.3.3 Non-enzymatic activation of CCl$_4$ by haem

The observation that the chemical reductant sodium dithionite could fully replace NADPH in promoting the reductive CCl$_4$-dependent inactivation of microsomal haem (see 3.3.1.1) raised the possibility that a direct reduction of cytochrome P-450 haem could result in CCl$_4$ activation even without involvement of the flavoprotein NADPH-cytochrome P-450-reductase. In order to test this possibility non-enzymic protohaem, as MHA, was incubated anaerobically with CCl$_4$ in presence of sodium dithionite and in the absence of microsomes, and the fate of haem was investigated under a number of conditions.

3.3.3.1 Haem undergoes rapid CCl$_4$-dependent inactivation

Preliminary experiments showed a marked, CCl$_4$-dependent modification of the pyridine/haemochrome spectrum suggesting that haem itself can be inactivated when incubated anaerobically with CCl$_4$ and sodium dithionite. Table 7 shows the results of a typical experiment.

An 84% loss of haem was observed on addition of CCl$_4$/dithionite but not when dithionite or CCl$_4$ were omitted. In this experiment dithionite alone was also responsible for some loss (8%) of haem.

In order to investigate whether the CCl$_4$-dependent loss of haem
Table 7. CCl₄-dependent loss of haem during anaerobic incubation of methaemalbumin with sodium dithionite.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Haem nmol/ml</th>
<th>(% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>3.76 ± 0.06</td>
<td>(100)</td>
</tr>
<tr>
<td>CCl₄ and dithionite</td>
<td>3.47 ± 0.06</td>
<td>(92)</td>
</tr>
<tr>
<td>CCl₄ and dithionite</td>
<td>0.61 ± 0.06</td>
<td>(16) *</td>
</tr>
</tbody>
</table>

NS, not significant and *, p < 0.001, when compared to corresponding values obtained in the absence of CCl₄ (Student's t test). MHA (9.8 nmol) was injected into 2.5 ml of the usual anaerobic buffer in rubber-stoppered glass tubes. At time 0, sodium dithionite (2.9 mM, final concentration) and/or CCl₄ (1 mM, final concentration) were injected into the incubation mixture. A control incubation was also performed in the absence of dithionite and CCl₄. After 5 minutes of incubation at 20°C 0.8 ml of the incubation mixture were added to pyridine/NaOH for haem assay. Values are mean ± SD of 3 determinations.
observed with the non-enzymic system resembled that obtained with microsomes, the porphyrin fluorescence method (see 3.2.4.1) was used together with the classical pyridine-haemochrome assay (see 3.2.2) to measure the haem loss in the same incubation. The porphyrin fluorescence technique was also used in the same experiment to investigate the protective effect of CO. As shown in Table 8, an equimolar loss of haem and protoporphyrin IX was found in this experiment, indicating that also with the chemical system haem underwent modification of its tetrapyrrolic structure. In this experiment some loss of haem and protoporphyrin IX was observed with dithionite alone. The loss, however, was much less marked than that observed in presence of both CCl₄ and dithionite, and it was not present consistently (little or no significant loss was seen, for example, in the experiments of Tables 7 and 9, respectively). The dithionite-dependent loss of haem was probably due to incomplete removal of oxygen from the buffer used to prepare the sodium dithionite solution.

As with microsomes, also in this experiment where the non-enzymatic system was used, CO prevented the CCl₄-dependent inactivation of haem. This suggested that a free ligand site on the haem iron was required for the activation of CCl₄. Further evidence for a critical role of the haem iron is described below.
Table 8. CCl₄-dependent loss of haem and protoporphyrin IX and effect of CO during anaerobic incubation of methaemalbumin with sodium dithionite.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Haem nmol/ml (% of control)</th>
<th>Protoporphyrin IX nmol/ml (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>2.24 ± 0.02 (100)</td>
<td>2.45 ± 0.13 (100)</td>
</tr>
<tr>
<td>CCl₄ dithionite</td>
<td>2.25 ± 0.04 (100) NS</td>
<td>2.44 ± 0.06 (100) NS</td>
</tr>
<tr>
<td>CCl₄/dithionite</td>
<td>1.74 ± 0.02 (78)</td>
<td>1.12 ± 0.02 (46)</td>
</tr>
<tr>
<td>CCl₄/dithionite + CO</td>
<td>£</td>
<td>1.35 ± 0.10 (55) $</td>
</tr>
</tbody>
</table>

NS, not significant and *, p < 0.001, when compared to corresponding values obtained in the absence of CCl₄; $, p < 0.001, when compared to the corresponding value obtained in the absence of CO (Student's t test). £, the CO interfered with the pyridine-haemochrome reaction (see 3.2.2.1). In all incubations MHA (containing 6.2 nmol haem) was added to 2.5 ml of the usual anaerobic buffer and, at time 0, CCl₄ (1 mM, final concentration) and/or sodium dithionite (1 mM, final concentration) were added. After a 5 min incubation 0.8 ml of incubation mixture were used for the pyridine-haemochrome assay and 110 ul were added to saturated oxalic acid for protoporphyrin IX determination. Values are mean ± SD of 3 determinations.
3.3.3.2 Protoporphyrin IX is not the site nor a target of CCl₄ activation

When authentic protoporphyrin IX, the iron-free precursor of haem, was complexed with human albumin, using the same procedure used to prepare MHA, and the porphyrin-albumin complex so obtained was incubated anaerobically with CCl₄ and sodium dithionite no porphyrin loss was observed (data not shown). These experiments provided convincing evidence that protoporphyrin IX, unlike haem, is unable to activate CCl₄ and to undergo "suicidal" CCl₄-dependent inactivation. These findings did not exclude, however, the possibility that the porphyrin itself might interact with CCl₄ reactive metabolites formed elsewhere, also resulting in a modification of the tetrapyrrolic structure.

In order to investigate this possibility, i.e. whether CCl₄ reactive metabolites formed by haem may attack and irreversibly modify protoporphyrin IX, equimolar amounts of haem and protoporphyrin IX, both as their water soluble complexes with human albumin, were incubated together anaerobically in the presence of CCl₄/dithionite. As reported in Table 9 no loss of protoporphyrin IX was observed in CCl₄/dithionite incubations, which on the other hand showed the expected loss of the pyridine-haemochrome reaction.
Table 9. Loss of haem, but not protoporphyrin IX, during their concurrent incubation with CCl₄/sodium dithionite.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Haem nmol/ml</th>
<th>Protoporphyrin IX nmol/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>2.59 ± 0.03</td>
<td>2.45 ± 0.03</td>
</tr>
<tr>
<td>CCl₄</td>
<td>2.60 ± 0.02</td>
<td>2.36 ± 0.02 NS</td>
</tr>
<tr>
<td>dithionite</td>
<td>2.53 ± 0.06</td>
<td>2.40 ± 0.02</td>
</tr>
<tr>
<td>CCl₄ and dithionite</td>
<td>0.55 ± 0.04 *</td>
<td>2.35 ± 0.07 NS</td>
</tr>
</tbody>
</table>

NS, not significant and *, p < 0.001, when compared to corresponding values obtained in the absence of CCl₄ (Student's t test). Experimental conditions were as for the experiment of Table 8. At the end of the 5 min incubation haem was measured by the usual pyridine haemochrome assay and protoporphyrin IX by the perchloric acid method by pipetting 0.3 ml incubation mixture into 3 ml methanol/1N-HClO₄ (1:1, v:v) for fluorescence determination. Values are mean ± SD of 3 determinations.
These results indicate that the haem iron is required not only for the activation of \( \text{CCl}_4 \) but also for the modification of the porphyrin ring and provide circumstantial evidence to suggest that the process of haem inactivation by \( \text{CCl}_4 \) is a suicidal process where each molecule of haem inactivated appears to be both the site of formation and the target of the same \( \text{CCl}_4 \) reactive metabolite. Further evidence for a suicidal mechanism of reductive haem inactivation by \( \text{CCl}_4 \) was obtained incubating \( [^{14}\text{C}] \)-haem with unlabelled \( \text{CCl}_4 \) or \( [^{14}\text{C}] \)-\( \text{CCl}_4 \) with unlabelled haem. These experiments will be described later in this thesis (5.3.1, 5.3.2 and 5.3.3).

3.3.3.3 \( \text{CCl}_4 \)-dependent loss of haem during concurrent, anaerobic incubation of liver microsomes and methaemalbumin

Following the observation that haem undergoes extensive destruction when incubated anaerobically with \( \text{CCl}_4 \) and the chemical reductant sodium dithionite, it was interesting to know whether in a biological system and in presence of a biological reductant haem was still able to catalyse its own inactivation. In preliminary experiments, therefore, a mixed system containing both microsomes and exogenous protohaem (as methaemalbumin) was incubated with \( \text{CCl}_4 \) in presence of NADPH and the \( \text{CCl}_4 \)-dependent loss of haem was studied.
Table 10. Loss of haem during incubation of NADPH-reduced microsomes and/or methaemalbumin (MHA) with CCl₄.

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Control (A)</th>
<th>+ CCl₄ (B)</th>
<th>Haem loss (A-B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. microsomes</td>
<td>3.76 ± 0.03</td>
<td>2.53 ± 0.03 *</td>
<td>1.23</td>
</tr>
<tr>
<td>2. MHA</td>
<td>2.65 ± 0.03</td>
<td>2.60 ± 0.06 NS</td>
<td>0.05</td>
</tr>
<tr>
<td>3. microsomes + MHA</td>
<td>6.06 ± 0.11</td>
<td>4.41 ± 0.09 *</td>
<td>1.65 **</td>
</tr>
</tbody>
</table>

NS, not significant and *, p < 0.001, when compared to control values. ** p < 0.01 when compared to values of incubation 1 (Student's t test). Values are mean ± SD of 3 determinations.

Microsomal protein (2.5 mg, 2.6 nmol cytochrome P-450/mg) from PB-treated rats was incubated at 21°C for 5 min together with 6.5 nmol haem (as MHA) in 2.5 ml of the usual anaerobic buffer containing 2.5 umol NADPH. The reaction was started by the addition to the incubation mixture of 2.5 umol CCl₄. The reaction was terminated by pipetting 0.8 ml incubation mixture into pyridine/NaOH for haem assay.
As shown in Table 10, no significant haem loss was observed, as expected, with MHA alone (incubation 2). On the contrary, the loss of haem measured at the end of incubation 3 was significantly greater than that of incubation 1 containing microsomes but not MHA. This unexpected result raised the possibility that exogenous haem might be able, even when NADPH was used as the reducing agent, to activate CCl₄ and catalyse its own suicidal destruction. In order to test this hypothesis a different mixed system was set up to quantitate microsomal and exogenous haem separately. Previous observations indicated (results not shown) that non-physiological haem analogues, like mesohaem and deuterohaem, were also able to undergo rapid degradation when complexed with human albumin and incubated anaerobically with CCl₄/dithionite. These analogues have different side chains and show different absorption spectra in pyridine/NaOH from those of protohaem. It was therefore possible to set up a spectrophotometric method which allowed the concurrent determination of protohaem and mesohaem in the same incubation mixture (see 3.2.3). The results of this time-course experiment are reported in Fig. 18.

A loss of approximately 50% of both proto- and mesohaem was observed in 5 min after the addition of CCl₄ to the microsomal suspension. The loss of protohaem predominated at earlier time points while that of mesohaem became evident only after 1 or 2 min.
Fig. 18. Loss of cytochrome P-450 protohaem (●) and exogenous mesohaem (○) during the combined incubation of NADPH-reduced microsomes and exogenous mesohaem with CCl₄. The anaerobic incubation contained 1.4 mg liver microsomal protein from PB-treated rats (1.95 nmol cytochrome P-450/mg), 4.2 nmol mesohaem (as MHA) and 2 umol NADPH in 2 ml of the usual anaerobic buffer. At time 0, 2 umol CCl₄ was added to test but not to control incubations to start the reaction. At the end of various incubation times up to 10 min, 0.8 ml incubation mixture were pipetted into pyridine/NaOH for concurrent spectrophotometric protohaem and mesohaem determination.
and became proportionately equal to or even greater than that of protohaem after a 10 min incubation. These findings suggested that mesohaem added to anaerobic microsomal suspensions may have undergone reduction, possibly by interaction with microsomal NADPH-cytochrome P-450 reductase in some way, and then interacted with CCl₄ in a "suicide" type of reaction. A number of experiments with either microsomes or purified NADPH-cytochrome P-450 reductase were carried out to test this hypothesis.

3.3.3.4 CCl₄-dependent loss of haem catalysed by NADPH-cytochrome P-450 reductase

Direct evidence for reduction of exogenous haem by both microsomes and purified NADPH-cytochrome P-450 reductase was obtained in the two following experiments. In one case, microsomes and oxidised haem (as MHA) were incubated together in CO-saturated buffer. The increasing formation of the CO-reduced haem complex observed on addition of NADPH was used to demonstrate the reduction of haem (Fig 19).

In all spectra two distinct peaks were observed. A 450 nm peak which was attributed to a CO-cytochrome P-450 complex, reached its maximal amplitude in a few minutes and remained substantially unchanged thereafter. The second peak observed, with a maximum initially at approximately 423 nm, was formed slowly and increased
Fig. 19. Reduction of exogenous haem by NADPH-supplemented rat liver microsomes as shown by the formation of a CO-haem difference spectrum. Exogenous haem (8.6 nmol), as MHA, was incubated at 22°C in each of two rubber-stoppered glass cuvettes together with 1.27 mg liver microsomal protein (1.62 nmol cytochrome P-450/mg) from PB-treated rats in approx. 2 ml of the usual anaerobic buffer. After recording the baseline between 500 and 400 nm (___) 20 ul of a 3 mM solution of K₃Fe(CN)₆ in 0.1 M Na₂HPO₄ buffer, pH 7.4, were added to both cuvettes (30 uM, final concentration) and the baseline was checked (---). Then both cuvettes were saturated with CO (1 bubble/min for 1 min) and, at time 0, 20 ul of 0.1 M NADPH were added to the test cuvette (1 mM final concentration) before recording the CO/reduced minus CO/oxidised difference spectrum at time 1, 10, 30, 60 and 90 minutes (___). In order to insure full reduction of all haem present in the test cuvette, 50 ul of 0.29 M sodium dithionite were added a few minutes before recording the final scan (...).
steadily for the subsequent 90 min, when the maximum was at 421 nm. Addition of excess sodium dithionite to the test cuvette at this point in time did not produce any further significant change in the wavelength maximum of this peak nor in the amplitude of either peaks. A likely explanation for this second peak is the progressive formation of a CO-haem complex upon gradual reduction of haem in solution by microsomal NADPH-cytochrome P-450 reductase.

A minor contribution to this peak at early times by reduced cytochrome $b_5$ is likely. As the concentration of cytochrome $b_5$ in these microsomes was about 0.50 nmol/mg protein, the incubation contained approximately 0.31 nmol cytochrome $b_5$/ml. Based on the $\epsilon_{\text{M}} \cdot \text{cm}^{-1}$ of 424-490 (for the reduced against oxidised difference spectrum of cytochrome $b_5$) of 112, the contribution of cytochrome $b_5$ to the 421 nm peak was calculated to be of approximately 10%. These data are consistent with the original hypothesis that NADPH-reduced microsomes can transfer electrons to haem in solution and point to NADPH-cytochrome P-450 reductase as the likely electron donor. In agreement with this interpretation, purified NADPH-cytochrome P-450 reductase incubated with MHA in the absence of microsomes was capable to catalyse the reduction of haem, as shown in the second experiment (Fig. 20).
Fig. 20. Reduction of exogenous haem by NADPH-cytochrome P-450 reductase as shown by the formation of a CO-haem difference spectrum. Initial incubation conditions were as for Fig. 19 except that both cuvettes contained 11.2 nmol haem, as MHA, but no microsomal protein, in 2.5 ml anaerobic buffer. After recording the baseline between 600 and 380 nm (---), no significant change of the difference spectrum was observed upon saturation of the sample cuvette with CO (----) nor when NADPH (1mM, final concentration) was added to the same cuvette while reference cuvette was saturated with CO (------). At time 0, 18.5 ug of a 0.37 mg/ml stock solution of purified flavoprotein was added to the sample cuvette and scans were recorded at 1, 5, 20 and 120 minutes. After the last scan 2.5 umol sodium dithionite was added to the sample cuvette 2 min before recording the final scan (...).
Upon addition of the flavoprotein to the incubation mixture a major peak at about 418 nm and two minor peaks at about 530 and 568 nm were increasingly formed in the CO/reduced minus CO/oxidised difference spectrum. These peaks were interpreted as the Soret and the $\alpha$ and $\beta$ peaks of the CO-haem complex formed by reduction of soluble MHA by NADPH-cytochrome P-450 reductase.

If this interpretation was correct one would expect that the addition of CCl$_4$ to this or similar incubation would result in the activation of the haloalkane and consequent loss of haem by the usual suicidal mechanism. Evidence showing that this is in fact the case was provided by the experiment described in Fig. 21.

As expected, a significant loss (>70% of initial value) of haem was observed after two hours in the complete incubation mixture (MHA/CCl$_4$/NADPH/flavoprotein). The haem loss was markedly slower than that observed with the non-enzymic system (MHA/CCl$_4$/sodium dithionite). Only approximately 30% of the haem initially present in the incubation mixture was lost in 10 min in contrast with 80% in the non-enzymic system (see 3.3.3.2). Interestingly, some loss of haem was also observed in this experiment in the absence of the reductase, but this took place more gradually than in presence of the reductase and was also much less marked.
Fig. 21. CCl₄-dependent haem loss catalysed by NADPH-cytochrome P-450 reductase. Haem (10.7 nmol, as MHA) was incubated at 22°C with 18.5 μg purified flavoprotein and 1 mM NADPH in 2.5 ml of the usual anaerobic 0.1 M Na₂HPO₄ buffer, pH 7.4. At time 0, 2.5 μmol CCl₄ was injected in the anaerobic incubation mixture to start the reaction. Control incubations contained NADPH or NADPH + CCl₄ but no reductase. At the indicated times the reaction was terminated and haem was measured by the pyridine/haemochrome assay.

* p < 0.01, + p < 0.001 in comparison with control values.
3.4 DISCUSSION

The metabolic activation of CCl₄ to reactive intermediates by the microsomal cytochrome P-450 system is a prerequisite for the toxic effects produced both in vivo and in vitro by this compound as reported in more detail in General Introduction (see 1.2.2). It has been known for some time that during CCl₄ metabolism cytochrome P-450 itself, but not NADPH-cytochrome P-450 reductase, the other component of the microsomal monooxygenase system, is inactivated (Sasame et al., 1968; Ota et al., 1975). This observation per se indicated a rather selective effect of CCl₄ reactive metabolites on the haemoprotein.

There are two major mechanisms considered to be responsible for the toxicity of CCl₄ in general, and for the CCl₄-dependent destruction of cytochrome P-450 in particular: the CCl₄-induced lipid peroxidation of the endoplasmic reticulum and the covalent binding of CCl₄ reactive metabolites to microsomal protein and lipid. The relative contributions of these two mechanisms to CCl₄-dependent toxicity may be determined by the experimental conditions and a number of factors such as the local concentration of oxygen and antioxidants, the form and concentration of cytochrome P-450, the activity of the flavoprotein, NADPH-cytochrome P-450 reductase, and its interaction with cytochrome P-450, etc. (Slater, 1982).
Certainly, the peroxidative degradation of the endoplasmic reticulum cannot be responsible for the inactivation of cytochrome P-450 and the loss of cytochrome P-450-dependent enzymatic activities observed in vitro during the anaerobic incubation of NADPH- or sodium dithionite-reduced microsomes with CCl₄ (Masuda, 1981; de Groot & Haas, 1981). Since the loss of the haemoprotein observed under these conditions was accompanied by an equivalent loss of microsomal haem, measured by the pyridine/haemochrome reaction, it was suggested that cytochrome P-450 may be damaged by a direct attack of CCl₄ reactive metabolites on its prosthetic group (De Toranzo et al., 1975; de Groot & Haas, 1981). The parallel, equimolar loss of microsomal haem and cytochrome P-450 observed in the present study (Fig. 14) indicates that, under the conditions of the experiment, the loss of haem was the only significant cause of enzyme inactivation, thus providing additional evidence in favour of the hypothesis mentioned above.

The loss of porphyrin fluorescence, which accompanied the disappearance of haem in almost stoichiometric amounts, indicates that the loss of haem as measured by the pyridine-haemochrome reaction could not be simply due to reversible masking of the haem iron by CCl₄ metabolites. It suggests instead that the tetapyrrolic structure of haem was also modified, probably by irreversible interaction with CCl₄ reactive metabolites. Evidence for the
covalent binding of \( \text{CCl}_4 \) reactive metabolites to haem-derived products will be reported in another part of this thesis (see 5.3.3 and 5.3.4).

The fluorescence technique of porphyrin measurement is not subjected to interference by high concentrations of \( \text{CO} \), like the usual method of haem measurement, the pyridine-haemochrome reaction, and it can be used, therefore, to investigate the protective effect of \( \text{CO} \) on \( \text{CCl}_4 \)-dependent haem loss. The protection against the haem loss by \( \text{CO} \) was strong but incomplete, despite the fact that a high concentration of inhibitor and strictly anaerobic conditions were used. Furthermore, lower concentrations (50 uM) of \( \text{CO} \) did not provide any significant protection against \( \text{CCl}_4 \)-dependent loss of cytochrome P-450. These results are in agreement with those reported by de Groot & Haas (1981) who found no protection against cytochrome P-450 loss with 10 uM \( \text{CO} \) and only approximately 64% protection with 1 mM \( \text{CO} \). The inhibition of the haem loss by \( \text{CO} \), although incomplete, is consistent with the hypothesis that \( \text{CCl}_4 \)-dependent haem destruction requires the interaction of \( \text{CCl}_4 \) with a reduced haem iron. This interaction probably involves transfer of electrons from the haem to the substrate. Interaction of \( \text{CO} \) with the reduced haem iron will make the electron transfer to \( \text{CCl}_4 \) impossible.

So, haem is not only the target but also the site of \( \text{CCl}_4 \) activation in cytochrome P-450. This view seems to be supported by
the additional finding that haem itself can catalyse its own CCl₄-dependent suicidal destruction in a purely chemical system where haem in solution as methaemalbumin plays the role of the suicidal activator. In this system, as with microsomes, the loss of haem was due to loss of its porphyrin tetrapyrrolic structure. The observation that protoporphyrin IX, the iron-free physiological precursor of haem was unable either to catalyze the reaction or be a target of CCl₄ activation, provided additional evidence to indicate that the reaction requires the haem iron as the site of CCl₄ activation and that the inactivation is probably confined to the same haem moiety. Further evidence suggesting an intramolecular type of mechanism for the destruction of haem will be presented later in this thesis (see 5.3.4).

A third mechanism of suicidal haem inactivation was observed, in the present study, on addition of CCl₄ to a "mixed" system in which exogenous haem compounds, whether proto- or mesohaem, were added to anaerobic NADPH-reduced microsomes. The involvement of the flavoprotein NADPH-cytochrome P-450 reductase in this process was investigated using purified flavoprotein. It was shown that the inactivation reaction was mediated by direct transfer of electrons from the flavoprotein to haem in solution. This process is similar to the transfer of reducing equivalents from NADPH-cytochrome P-450 reductase to cytochrome c which occurs in the assay system for the
measurement of the reductase activity. Haem reduction probably results in reductive activation of \( \text{CCl}_4 \) and consequent loss of haem. A mechanism for the suicidal, NADPH-cytochrome P-450 reductase-dependent activation of \( \text{CCl}_4 \) by haem is proposed in General Discussion (see 6.)

A good agreement was observed between inactivation of cytochrome P-450 haem (Table 4) and metabolism of \( \text{CCl}_4 \) (Table 1, see 2.3.5.) with liver microsomal preparations obtained from rats treated with different inducers. Pretreatments of animals which increased the production of \( \text{CCl}_2 \) and CO from \( \text{CCl}_4 \) were also responsible for a greater loss of cytochrome P-450, both in absolute amounts and as a percentage of total haemoprotein initially present. The high loss of cytochrome P-450 haem found with PB microsomes in the present study is consistent with the observation that a rat cytochrome P-450 isoenzyme specifically induced by PB seems to be selectively responsible for and destroyed by the \text{in vivo} \) formation of \( \text{CCl}_4 \)-derived reactive intermediates, such as \( \cdot \text{CCl}_3 \) (Noguchi et al., 1982a and b). Interestingly, however, some loss of microsomal haem was also observed with control or \( \beta \)-NF microsomes indicating that significant amounts of \( \text{CCl}_4 \)-susceptible forms of cytochrome P-450 were also present in these microsomes.

It has been shown in another part of this thesis (see 2.3.3) that the 460 nm peak observed in the difference spectrum of anaerobically
reduced liver microsomes on addition of CCl$_4$, is probably due to the formation of a carbene-cytochrome P-450 complex. It has been already mentioned that stable carbene-iron-porphyrin complexes are formed upon reductive interaction of CCl$_4$ with synthetic porphyrins, for example Fe(II)-tetraphenylporphyrin. An important role of the carbene in cytochrome P-450 destruction and other aspects of CCl$_4$ toxicity has been proposed (Lange & Mansuy, 1981; Nastainczyk et al., 1982). It has been suggested (de Groot & Haas, 1981) that the haem moiety of cytochrome P-450 might be alkylated by :CCl$_2$ resulting in the inactivation of the haemoprotein. However, if :CCl$_2$ were to play a significant role in the process, one would expect that its trapping by the specific carbene-trapping agent DMB should lead to inhibition of the CCl$_4$-dependent haem loss. The lack of any significant effect of DMB observed in the present study (Table 5) strongly suggests that :CCl$_2$ is not significantly involved in the destruction of haem indirectly indicating that ·CCl$_3$ may be the reactive species responsible for cytochrome P-450 haem inactivation.

A number of conditions have to be fulfilled by an enzyme inhibitor in order to be classified as a suicide substrate. According to Rando (1974) three factors are crucial to the successful design of suicide inhibitors. First of all the enzyme must activate "a chemically unreactive molecule to a reactive one".
Secondly, the reactive intermediate "must be generated within bonding distance of a crucial active site residue". The third condition is that the reactive molecule must form a covalent bond with the enzyme resulting in its irreversible (over the time scale of the experiment) inactivation. In most cases, however, not all catalytic cycles result in enzyme inactivation since the enzyme-inhibitor complex can follow two distinct routes. It can either dissociate or it can result in a covalent bond. The higher the ratio between rate of covalent binding and that of dissociation, the more effective will be the inhibitor.

Pseudo first-order kinetics are generally reported for enzyme inactivation reactions by "suicide" substrates (Walsh et al., 1978; Waley, 1980; Ortiz de Montellano & Mico, 1981; Loosemore et al., 1981). The inactivation, however, is not exactly first-order and the extent of deviation depends on the rate by which the substrate concentration changes during the reaction (Waley, 1980). A number of general schemes for the kinetic mechanism of enzyme inactivation by suicide substrates has been reported (Rando, 1974; Walsh et al., 1978; Waley, 1980). These schemes are substantially similar and can be summarised as follows:
where $E$ is the enzyme, $S$ the substrate, $P$ the product, $ES$ the Michaelis enzyme-substrate complex, $EP$ a second intermediate and $E_i$ the inactivated enzyme.

As discussed above, some enzyme-substrate complexes $EP$ undergo substrate turnover reaction ($k_3$) and some other enzyme inactivation ($k_4$). The ratio $k_3/k_4$ will represent the number of catalytic events corresponding to each enzyme inactivation event. This ratio (commonly referred to as "partition ratio", Waley, 1980) is independent of substrate or enzyme concentration and is a characteristic parameter indicative of the "effectiveness" of suicide substrates: the lower the partition ratio the more effective the suicide substrate (Walsh C., 1977; Walsh et al., 1978). As noted by Waley (1980), depending on the value of the partition ratio and the relative initial concentrations of enzyme and substrate in the incubation mixture, the reaction will only terminate in one of two possible ways. (A), if the partition ratio is smaller than the ratio between actual substrate and enzyme concentrations, the reaction

\[ (7) \quad E + S \xrightleftharpoons[k_{-1}]^{k_1} ES \xrightarrow{k_2} EP \xrightarrow[k_3]{k_4} E + P \]
will come to an end when all the enzyme is inactivated. (B), if the partition ratio is greater, the reaction will be terminated when no more substrate is available. In this case all the substrate present initially will be metabolised but some of the enzyme will still be active. It follows that, knowing a) which component is limiting in the reaction and b) the initial concentrations (or amounts) of both components and the final concentration (or amount) of the component which is not limiting, the partition ratio can be calculated for that particular substrate-enzyme association. This method was used to calculate the partition ratio between CCl$_4$ metabolism and CCl$_4$-dependent haem loss (Table 6).

Using liver microsomes from Aroclor-treated rats and assuming that one single CCl$_4$ reactive metabolite is sufficient for cytochrome P-450 haem destruction, a value of about 26-27 was found for the partition ratio between CCl$_4$ turnover and cytochrome P-450 inactivation. This value is approximately one order of magnitude smaller than that reported for another suicide substrate, allylisopropylacetamide (Ortiz de Montellano & Mico, 1981; Loosemore et al., 1981). This indicates that CCl$_4$ is remarkably effective as a suicide substrate of cytochrome P-450.
4. Suicidal inactivation of haem by halothane

4.1 INTRODUCTION

The anaesthetic halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) undergoes both oxidative and reductive metabolism by cytochrome P-450. The oxidative pathway results in the formation of the stable end product trifluoroacetic acid (Stier, 1964; Rehder et al., 1967) while the reductive metabolism leads to the formation of free radical (Fujii et al., 1984; Tomasi et al., 1983) and carbanion (Ahr et al., 1982) intermediates. The reactive intermediates formed may undergo further reactions to form 2-chloro-1,1-difluoroethylene (CDE) and 2-chloro-1,1,1-trifluoroethane (CTE), two volatile metabolites detected in the expired air of animals and man exposed to halothane (Mukai et al., 1977; Sharp et al., 1979). The reductive pathway is considered to be responsible for the hepatotoxicity observed in animals treated with halothane under low oxygen tension (Jee et al., 1980).

During the anaerobic incubation of halothane with rat liver microsomes supplemented with NADPH or sodium dithionite, the following effects were reported: (1) a typical difference spectrum with a 470 nm peak (Nastainczyk et al., 1978; Baker et al., 1983), (2) production of inorganic fluoride (Maiorino et al. 1981; Van Dyke & Gandolfi, 1976) and (3) inactivation of cytochrome P-450 and loss
of haem, the prosthetic group of the cytochrome (Krieter & Van Dyke, 1983). It has been reported that the anaerobic incubation of halothane with boiled microsomes or haemin in presence of the chemical reductant sodium dithionite resulted also in the formation of inorganic fluoride (Baker et al., 1983). Moreover heat-denatured hepatic microsomes were able to convert halothane to CTE in presence of sodium dithionite (Ahr et al., 1982). These results indicate that the reductive dehalogenation of halothane does not require the protein portion of cytochrome P-450 and that haem itself in the absence of the apoprotein can act as a catalyst.

The evidence to be described below supports the hypothesis of a non-enzymatic mechanism of halothane activation by haem which shares several features of a typical suicide inactivation reaction. The suicidal haem loss reported here resembles that already described for another polyhalogenated chemical, carbon tetrachloride, which was found to be responsible for a dramatic, suicidal loss of the haem catalyst (see 3.3.3). The similar results obtained with the two compounds are consistent, as a whole, with a common mechanism of non-enzymatic, reductive activation of some polyhalogenated alkanes by haem.
4.2 MATERIALS AND METHODS

4.2.1 Chemicals and biochemicals

Halothane was purchased from Aldrich Chimica, Milano, Italy. Isoflurane and enflurane, both from Abbott SpA, Aprilia (Latina), Italy, were a gift of Dr. Tegazzin (O.O.T., Padova, Italy). NADPH, human albumin, catalase (EC 1.11.1.6), glucose oxidase (EC 1.1.3.4) and hemin were from Sigma Chemical Co., St. Louis, MO, U.S.A. Sodium fluoride standard (0.1 M) and total ionic strength solution (TISAB) were purchased from Orion Research Inc. (Boston, MA, U.S.A.). All other chemicals were of either analytical grade or the best quality available. MHA was prepared according to the method of Tenhunen et al., (1968) as described in 2.2.1.

4.2.2 Microsomal and mitochondrial preparations and incubations

Microsomes were prepared from male Wistar Albino rats (170-210 g) obtained from the Institute of General Pathology Animal House (University of Padua, Padova, Italy) and pretreated i.p. with phenobarbital (PB, 80 mg/kg b.w., daily for 3 days) as described in 2.2.2. Mitochondria, prepared from the liver of untreated Wistar Albino rats by the method of Johnson and Lardy (1967) were provided by Dr. A. Bindoli (Institute of Biological Chemistry, University of Padua, Padova, Italy). All incubations were performed anaerobically.
as described in 2.2.7. The haloalkanes were added to incubations using the minimum amount of methanol as solvent.

4.2.3 Assays

Haem was measured either by the usual pyridine/haemochrome method of Paul et al. (1953) or by the fluorescence technique of Morrison (1965) as described in 3.2.2 and 3.2.4.1, respectively. Inorganic fluoride was measured in the incubation mixture after dilution of the mixture with Orion TISAB buffer, using a fluoride-specific electrode (Orion, model 94-09) and a model 90-01 reference electrode. Calibration curves were prepared immediately before measurements, as described by Orion (Model 94-09, 96-09 Fluoride/Combination Fluoride Electrodes Instruction Manual, Boston, MA, U.S.A., 1987) using NaF solutions of known concentrations prepared from the Orion 0.1 M standard diluted in an equal volume of TISAB buffer. The lower level of detection of inorganic fluoride under these conditions was 0.5 uM.

4.3 RESULTS

4.3.1 Production of inorganic fluoride and loss of haem during anaerobic incubation of halothane with methaemalbumin

On addition of halothane (1-10 mM, final concentration) to
carefully maintained anaerobic incubation mixtures containing 11.8 uM haem, as MHA, in presence of 1 mM sodium dithionite as the reducing agent, a dose-dependent reduced difference spectrum with a large peak at about 475 nm was observed (results not shown). In another group of experiments MHA was incubated with halothane or with isoflurane or enflurane - two other halogenated anaesthetics which, unlike halothane, are not metabolized to any significant extent by cytochrome-P-450-in the presence and absence of dithionite. Both the production of inorganic fluoride and the recovery of haem were measured in the same incubation mixtures. As shown in Table 11, only the incubation mixture containing both halothane and dithionite gave a measurable fluoride production and a significant haem loss, thus indicating that halothane metabolism and haem loss were associated.

4.3.2 Halothane-dependent loss of haem in incubations with methaemalbumin, liver microsomes or mitochondria

The reductive, halothane-dependent haem loss was compared in three different systems, i) MHA, ii) liver microsomes from PB-treated rats and iii) liver mitochondria from untreated rats. The concentrations of substrates and reducing agent and the incubation conditions were the same in the three systems. The results are shown in Table 12.
Table 11. Production of inorganic fluoride and loss of haem during the reductive incubation of methaemalbumin with different anaesthetics

<table>
<thead>
<tr>
<th>Addition</th>
<th>Fluoride (nmol/min/nmol haem)</th>
<th>Haem (nmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>ND</td>
<td>21.1</td>
</tr>
<tr>
<td>Sodium dithionite (D, 5 mM)</td>
<td>ND</td>
<td>22.6</td>
</tr>
<tr>
<td>D and halothane (5 mM)</td>
<td>4.8</td>
<td>6.8</td>
</tr>
<tr>
<td>D and isoflurane (5 mM)</td>
<td>ND</td>
<td>19.5</td>
</tr>
<tr>
<td>D and enflurane (5 mM)</td>
<td>ND</td>
<td>18.9</td>
</tr>
<tr>
<td>Halothane (5 mM)</td>
<td>ND</td>
<td>20.8</td>
</tr>
</tbody>
</table>

ND, not detectable. Incubations were for 10 min at 37° C and contained 23.7 nmol haem/ml and the indicated final concentrations of haloalkane and reducing agent in 2 ml anaerobic buffer. At the end of the incubation time 0.8 ml were pipetted into 1.87 ml pyridine/NaOH for haem determination and 1 ml into Orion TISAB buffer, after dilution with water (1:10, v:v), for fluoride measurement.
Table 12. Loss of haem during the anaerobic incubation of different anaesthetics with methaemalbumin, liver microsomes or mitochondria in the presence of sodium dithionite

<table>
<thead>
<tr>
<th>Addition</th>
<th>Haem</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MHA (nmol/ml)</td>
</tr>
<tr>
<td>None (control)</td>
<td>9.91±0.11</td>
</tr>
<tr>
<td>Dithionite (D, 5mM)</td>
<td>9.57±0.27 NS</td>
</tr>
<tr>
<td>D and halothane (5mM)</td>
<td>3.32±0.39 **</td>
</tr>
<tr>
<td>D and isoflurane (5mM)</td>
<td>10.25±1.27 NS</td>
</tr>
<tr>
<td>D and enflurane (5mM)</td>
<td>9.60±0.06 NS</td>
</tr>
</tbody>
</table>

NS, not significant; *, p < 0.01; **, p < 0.001 (Student's t test). Incubations were for 10 min at 37°C and contained either 23.7 nmol haem (MHA) or 1.5 mg microsomal protein (2.35 nmol cytochrome P-450) or 5.5 mg mitochondrial protein and 5 mM anaesthetic and reducing agent in 2 ml anaerobic buffer. At the end of the incubation haem was measured as indicated for Table 11. Values are mean ± SD of 3 determinations.
In incubations with methaemalbumin halothane in the presence of dithionite resulted in an approx. 70% loss of haem. No loss was observed with the other two anaesthetics, in presence or absence of dithionite, nor with halothane alone. In the microsomal system halothane, but not the other anaesthetics, gave a 40% loss of haem. This is consistent with the results reported by others (Krieter & Van Dyke, 1983). Qualitatively similar results were observed with mitochondria. In this case the halothane-dependent loss of haem was about 30%. In the absence of the reducing agent no significant loss of haem was found in any of the three systems with any of the substrates alone.

4.3.3 Kinetics of haem inactivation by halothane

The kinetics of haem inactivation by halothane are reported in Fig. 22. The semilog plot of haem concentration (as measured by the pyridine/haemochrome reaction) against time after addition of halothane shows a linear (first-order) decrease, as would be expected from a suicide type of reaction. The plot, however, is biphasic with the indicated half times of inactivation. Possible reasons for this are considered in the Discussion Section (see 4.4).
Fig. 22. Inactivation kinetics of haem from MHA by halothane. Incubation conditions were as for Table 13. The log % of haem (pyridine/haemochrome method) remaining in the incubation mixture at the indicated times after addition of halothane to the incubation mixture was plotted against time of incubation.
4.3.4 Halothane-dependent loss of haem and destruction of the tetrapyrrolic structure of haem; inhibition by carbon monoxide

In order to investigate whether the loss of haem with halothane was accompanied by an irreversible loss of the porphyrin ring, as for CCl₄, haem was measured in the same incubations by two different methods: the pyridine/haemochrome reaction and the porphyrin fluorescence method. Halothane in the presence of sodium dithionite was responsible for a significant loss of both constituents (Table 13).

The results of this experiment indicated that the tetrapyrrolic ring was irreversibly modified by halothane metabolites. Moreover, using the porphyrin/fluorescence method an almost 50% protection of the halothane-dependent loss of haem was observed with carbon monoxide, indicating that a free haem iron was required for halothane activation. Correction for catalase haem (0.16 nmol/ml) was only made for haem but not for protoporphyrin IX values in this experiment. This in part accounts for the slightly higher values obtained for protoporphyrin IX as compared to haem concentrations.

When the time course of haem inactivation was investigated using these two methods, haem and porphyrin loss were similar but not overlapping (Fig. 23). After 10 seconds from the addition of halothane to the dithionite-reduced incubation mixture containing
Table 13. Halothane-dependent loss of haem and protoporphyrin IX from MHA and the effect of carbon monoxide

<table>
<thead>
<tr>
<th>Addition</th>
<th>Haem (nmol/ml)</th>
<th>Protoporphyrin IX (nmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>9.3 ± 0.3 (100%)</td>
<td>11.8 ± 1.4 (100%)</td>
</tr>
<tr>
<td>Halothane (5mM)</td>
<td>9.1 ± 0.4 (98%)</td>
<td>11.2 ± 0.9 (95%) NS</td>
</tr>
<tr>
<td>Dithionite (D, 5mM)</td>
<td>8.9 ± 0.3 (97%)</td>
<td>9.5 ± 1.0 (81%) NS</td>
</tr>
<tr>
<td>D and halothane (5mM)</td>
<td>3.2 ± 0.1 (33%) **</td>
<td>2.7 ± 0.6 (23%) **</td>
</tr>
<tr>
<td>CO, D (5mM)</td>
<td>&amp;</td>
<td>6.4 ± 0.5 (54%) *</td>
</tr>
</tbody>
</table>

NS, not significant; *, p < 0.01; **, p < 0.001 (Student's t test). &; not measurable because of interference of CO with the assay (see 3.2.2.1). Incubations were for 5 min at 37°C and contained 11.8 nmol haem/ml as MHA and the indicated final concentrations of halothane and dithionite in 2 ml anaerobic buffer. At the end of the incubation time, 0.8 ml were added to pyridine/NaOH for haem measurement and 0.15 ml to saturated oxalic acid for porphyrin fluorescence determination using a calibration curve obtained with standard solutions of MHA. MHA in the oxalic acid method was known from previous experiments to give identical fluorescence values as authentic protoporphyrin IX (see 3.2.4.4). When indicated, CO was bubbled through the incubation mixture for 30 sec after the addition of dithionite and before halothane injection. Values are mean ± SD of 3 to 5 determinations.
Fig. 23. Loss of haem and protoporphyrin IX during reductive incubation of methaemalbumin with halothane. Incubation conditions were as for Fig. 22. At the indicated incubation times pyridine/haemochrome spectra and porphyrin fluorescence were determined as for Table 13.
MHA, a 25% loss of haem but no loss of porphyrin fluorescence was found.

At 1 minute incubation time the loss was about 50% for both haem and protoporphyrin IX, and at later times the loss of porphyrin fluorescence was apparently even greater than that of the pyridine/haemochrome reaction, but the difference was almost negligible.

4.4 DISCUSSION

It has been shown here that on addition of halothane to an anaerobic, cytochrome P-450-free system containing methaemalbumin and sodium dithionite as the reducing agent, haem is able (1) to give a difference spectrum, (2) to produce inorganic fluoride and (3) to catalyze its own destruction, thus mimicking all the effects observed with the microsomal haemoprotein.

The reduced difference spectrum observed on addition of halothane to sodium dithionite-reduced incubations of MHA resembled that obtained with CCl₄ under the same conditions, except that with halothane the maximum was at a slightly higher wavelength (475 and 470 nm, with halothane and CCl₄, respectively). The observation of this spectrum is at variance with the results of Baker et al. (1983) who observed a halothane-dependent difference spectrum with dithionite-reduced microsomes but not haemin. This could be due to
the fact that these workers used an approximately tenfold lower ratio of substrate to haem concentrations as compared with that used in the present study.

The halothane-dependent loss of haem observed in all three systems examined (methaemalbumin, microsome and mitochondria) indicate both enzymatic and non-enzymatic mechanisms of haem inactivation by halothane. Similar mechanisms of activation have also been described for CCl₄ in the earlier sections of this thesis. Haem in solution as methaemalbumin in the presence of a chemical reductant was shown to activate halothane and CCl₄ to reactive, haem-inactivating metabolites. Moreover with both compounds this non-enzymatic model system was shown to mimic microsomal cytochrome P-450 in many respects. It is not unreasonable, therefore, to predict for halothane and CCl₄ a common molecular mechanism of suicidal haem inactivation. In radioisotopic studies with ¹⁴CCl₄, for instance, it was found that one molecule of CCl₄ reactive metabolite binds and inactivates one molecule of haem (see 5.3.4). Therefore, a 1:1 stoichiometry between haem loss and reactive metabolites bound to haem products would also be expected with halothane. This of course needs further investigation.

Suicide inactivation reactions require the following conditions: (a) destruction of the suicidal catalyst, (b) pseudo first-order kinetics of inactivation and (c) protection by agents competing for
the active site but not by scavengers of the reactive species (Waley, 1980; Walsh et al., 1978; Loosemore et al., 1981). For details on the kinetics of suicide inactivation reactions see 3.4. The non-enzymatic mechanism of haem inactivation by halothane was found to fulfil condition (a) and (b) and, in part, condition (c). These aspects are considered in more detail.

(a) The loss of haem as measured by both the pyridine-haemochrome reaction and the porphyrin fluorescence technique indicates that the structure of haem has been irreversibly damaged.

(b) The kinetics of cytochrome P-450 destruction observed are compatible with a suicide enzyme inactivation reaction. The reason for the biphasic plot observed, however, is not known at the present time, but several possible explanations can be considered. One explanation may be the presence in the incubation mixture of two different pools of haem, possibly due to different modes of interaction with the albumin, resulting in different rates of access of the substrate to the iron atom active site. Alternatively, it is possible that two separate reactions, possibly involving different reactive metabolites, are responsible for the inactivation of haem. This interpretation is consistent with the findings of Gandolfi et al. (1980) who suggested that multiple reactive intermediates may form during microsomal activation of halothane. They found differences in the covalent binding of \(^{3}H\)-versus
[\textsuperscript{14}C]-halothane indicating that the hydrogen atom (\textsuperscript{3}H) is retained on some, but not all reactive intermediates of halothane.

Another explanation could be that, during the fast component of the reaction, metabolites of halothane bind to the haem, thus preventing the reaction of the pyridine with the haem iron, analogous to CO, and resulting in an apparently faster haem loss. The results represented in Fig 12 are consistent with this last possibility. The discrepancy between haem and porphyrin loss observed at early incubation times seems indeed to support the suggestion that a metabolite is initially formed which masks the haem iron but which does not modify the structure of the porphyrin ring. Furthermore, this interpretation is in agreement with what has been proposed to occur in microsomal incubations. Indeed the formation of a ligand complex between cytochrome P-450 and some halothane metabolites, probably cytochrome P-450 Fe\textsuperscript{3+}--\Theta CHClCF\textsubscript{3} carbanion complex, is thought to be responsible for the apparent loss of cytochrome P-450 observed during the anaerobic incubation of liver microsomes with halothane and NADPH or dithionite (Ahr et al., 1982).

(c) As far as the fulfillment of this condition is concerned, the protection provided by CO against the haem loss, although incomplete, indicates that a free, reduced haem iron is required for the activation of halothane to occur.
Based on the present results the following non-enzymatic mechanism of reductive haem inactivation by halothane is proposed. Halothane is activated non-enzymatically to reactive metabolites by haem in the presence of a reducing agent. These intermediates can either undergo further reductive dehalogenation resulting in the formation of stable metabolites such as inorganic fluoride or bind and irreversibly modify the haem moiety.
5. Radioisotopic experiments and isolation and partial characterization of CCl₄-dependent haem products

5.1 INTRODUCTION

The loss of cytochrome P-450 occurring during metabolic activation of carbon tetrachloride is known to be accompanied by both breakdown of the prosthetic haem moiety (Levin et al., 1972; Guzelian & Swisher, 1979) and loss of the apoprotein, the polypeptide portion of the enzyme (Noguchi et al., 1982b; Moody et al., 1986). Evidence for a causative role of the haem loss in the CCl₄-dependent destruction of cytochrome P-450 has been presented (Levin et al., 1972; Davies et al., 1985 and 1986), although the molecular mechanism and the products of haem degradation have not been yet identified.

During anaerobic incubation of NADPH- or sodium dithionite-reduced rat liver microsomes with CCl₄, equimolar amounts of protohaem and cytochrome P-450 were lost (de Groot & Haas, 1981), due to irreversible modification of its tetrapyrrolic structure as reported elsewhere in the present thesis (see 3.3.1.2). A rapid loss of haem has been also observed in the present study when MHA was incubated anaerobically with CCl₄ and sodium dithionite in a purely chemical system (see 3.3.3.1). This indicated that haem itself is capable of catalyzing CCl₄ activation in a suicidal manner even in
the absence of the apoprotein.

Here, the suicidal mechanism of haem inactivation by \( \text{CCl}_4 \) was investigated further using this non-enzymic system. After incubation of radiolabelled haem (as MHA) with unlabelled \( \text{CCl}_4 \) or radiolabelled \( \text{CCl}_4 \) with unlabelled haem in presence of dithionite, the resulting pigments were analysed by an h.p.l.c. technique. Moreover, the products of haem degradation were isolated by t.l.c. and partially characterized by their spectra in pyridine/NaOH. The results obtained provide additional, circumstantial evidence that the prosthetic haem moiety is both the site and the target of the suicidal activation of \( \text{CCl}_4 \) by cytochrome P-450 and offer insights in the molecular mechanism of this process.

5.2 MATERIALS AND METHODS

5.2.1 Chemicals and biochemicals

Methanol and tetrabutylammonium hydroxide (TBA) containing a phosphate buffer, pH 7.4, were both h.p.l.c. grade and were purchased from Rathburn Chemicals Ltd. (Walkerburn, Scotland) and Fisons (Loughborough, England), respectively. Water was made h.p.l.c. grade by a Milli.Q Water Purification System (Millipore Ltd., Harrow, England). Chloroform was made peroxide- and ethanol-free by 5 washes with equal volumes of water and was dried with
sodium sulphate immediately before use.

$[^{14}\text{C}]\text{-CCl}_4$ (4.0 mCi/mmol) and 5-amino$[^{14}\text{C}]$-laevulinic acid hydrochloride ($[^{5}\text{C}]$-ALA, 49 mCi/mmol) were purchased from New England Nuclear Research Products (Boston, MA, U.S.A.). 5-Amino$[^{4}\text{C}]$-laevulinic acid hydrochloride ($[^{4}\text{C}]$-ALA, 55 mCi/mmol) was purchased from Amersham International plc. (Amersham, Buckinghamshire, U.K.). All other chemicals were either of analytical grade or the best quality available.

Methaemalbumin (MHA) was prepared as described in 2.2.1 by complexing radiolabelled or unlabelled protohaem with human albumin. $[^{14}\text{C}]$-Haem from $[^{4}\text{C}]$-ALA was either prepared as described below or, together with $[^{14}\text{C}]$-haem from $[^{5}\text{C}]$-ALA, a gift of Dr. F. De Matteis (MRC Toxicology Unit, Carshalton, England).

5.2.2 Biosynthesis of $[^{14}\text{C}]$-haem

$[^{14}\text{C}]$-Haem was prepared biosynthetically from $[^{5}\text{C}]$-ALA or $[^{4}\text{C}]$-ALA using a chicken haemolysate incubation according to a modification of the method of Dresel and Falk (1954). The labelling pattern in the haem moiety is different with the two radiolabelled precursors (Fig. 24).

The procedure used for preparation of $[^{14}\text{C}]$-haem with $[^{4}\text{C}]$-ALA was the following. Approximately 27 ml of hen blood were collected, heparinised with 10 ml of a 0.9% NaCl solution containing a few
Fig. 24. Biosynthetic labelling of haem from [5-$^{14}$C]-ALA or [4-$^{14}$C]-ALA.
drops of a commercial heparin solution, filtered through tissue wet with 0.9% NaCl and the volume brought to 70 ml with 0.9% ice cold NaCl. After centrifugation at 3,000 rpm for 15 min the aqueous supernatant and the leucocyte layer was removed with a vacuum pump and the RBC remaining were washed twice with saline at 4°C. The cells were equally divided into two test tubes and cooled to 0°C for 10-15 min before adding 11 ml ice cold twice distilled water to each tube with hand stirring to haemolyse the RBC. After 5 min, 2.75 ml of 0.15 M Na₂HPO₄ buffer, pH 7.4, containing 0.60 M KCl were added to each tube to restore isotonicity. Haemolysates were mixed together and 18 ml of combined haemolysate were transferred to a 100 ml conical flask before adding the following: 3.3 ml of 36 mM MgCl₂, 3.3 ml of 10 mM FeSO₄ and 6.4 ml normal saline. The mixture was equilibrated for a few minutes at 38°C before the addition of a 2 ml ALA solution containing 87 nmol [4-¹⁴C]-ALA (5 uCi) and 1 umol cold ALA. The mixture was stoppered with cotton wool, mixed gently and incubated for 4 hours at 38°C and 30 double oscillations/min. At the end of the incubation time the incubation mixture was added gradually with vigorous stirring to 800 ml ethyl acetate/glacial acetic acid (4:1, v:v) and left to stand overnight at room temperature for protein precipitation. The haem solution was filtered through a sintered glass filter and the filtrate was extracted twice with 100 ml saturated sodium acetate solution. The sodium acetate extracts
were then extracted with approximately 100 ml ethyl acetate and the combined ethyl acetate extracts were washed as follows: twice with 200 ml 3% (w:v) sodium acetate in water, once with 500 ml 15% (v:v) HCl and once with 500 ml twice distilled water. This procedure allowed removal of any pre-existing or newly formed free porphyrins from the haem preparation. Finally, the ethyl acetate was removed under vacuum and the crude $^{14}$C-labelled haem residue was recovered in a 50 ml spherical flask.

$^{14}$C-Haem was recrystallized using the method of Labbe and Nishida (1957). The crude haem residue was dissolved in 0.5 ml pyridine, before the addition of 50 ml of a 3:1 (v:v) mixture of acetone with 2% (w:v) SrCl$_2$·6H$_2$O in glacial acetic acid. The mixture was mixed for 30 min, filtered through acetone-wet Whatman No. 1 filter paper and the acetone was evaporated by heating. The approximately 10 ml of solution remaining were transferred to a test tube and cooled on ice to produce the haem crystals. After centrifugation at 2,600 rpm for 10 min to sediment the crystals, the supernatant was decanted and the crystals were resuspended in 3 ml 50% glacial acetic acid, mixed and centrifuged as above. The precipitate was washed twice with 4 ml water and once with 2 ml ethanol and 4 ml ether, respectively. Finally, the ether was decanted and the haem crystals were dried in a desiccator containing silica gel.
Estimation of $^{14}$C-haem specific radioactivity was performed as follows. A few crystals were dissolved in about 1 ml pyridine, the solution was filtered through cotton wool in a Pasteur pipette and the filtrate collected in a teflon-stoppered glass tube. Then 50 ul of this filtrate were added in triplicate to 3 ml pyridine/0.2 N NaOH (1:1, v:v) and the reduced absolute spectrum was recorded in a dual beam UV-VIS spectrophotometer model Varian 2200. The haem concentration in the pyridine/NaOH solutions was calculated from the difference in absorbance between 557 and 541 nm (maximum and trough, respectively) in this spectrum and the extinction coefficient reported by Falk (1964a) (see 3.2.2). After correction for dilution, the mean ($\pm$ SD) haem concentration in the pyridine filtrate was finally obtained (0.213 ± 0.001 mM). Fifty ul of pyridine filtrate were added in triplicate to 4 ml MP scintillant in 4-ml minivials and the radioactivity in these minivials was counted. A blank of MP scintillant alone was also counted. After counting, 12.5 nCi (50 ul of a 0.25 uCi/ml solution of $^{14}$C-ALA in pyridine) were added as an internal standard to each minivial and the radioactivity in the minivials was recounted. The difference between the mean c.p.m. value obtained in the second counting after addition of the internal standard ($\text{cpm}\_\text{SPL+STD}^{\text{SPL}}$) and that obtained in the first counting ($\text{cpm}\_\text{SPL}^{\text{SPL}}$) both corrected for the c.p.m. value of the blank, was divided by the known d.p.m. value of the internal standard ($\text{dpm}\_\text{STD}^{\text{STD}}$).
to obtain the efficiency (E) as follows:

\[
E = \left( \frac{\text{cpm}_{\text{STD+SPL}} - \text{cpm}_{\text{SPL}}}{\text{dpm}_{\text{STD}}} \right) \times 100 = \left( \frac{18,680 - 622}{27,750} \right) \times 100 = 65\%
\]

By using this efficiency value and the mean c.p.m. value of the sample before the addition of the internal standard (cpm_{SPL} = 622 c.p.m.), a value of 957 d.p.m. was calculated for the haem sample, corresponding to 0.43 nCi. Knowing the nmoles of haem present in the 50 ul pyridine filtrate used for counting, the specific radioactivity of [\(^{14}\)C]-haem was calculated to be 0.0403 nCi/nmol. Since approximately 35 mg of crystals were obtained the total final radioactivity recovered as [\(^{14}\)C]-haem was 2.16 uCi, corresponding to 43% of the initial radioactivity (5 uCi) added to the haemolysate as [4-\(^{14}\)C]-ALA.

5.2.3 Haem methylation

Methylation of haem and haem products was performed, soon after each incubation, as follows. One volume (usually 1-2 ml) of incubation mixture was added dropwise to 10 volumes of an ice-cold, carefully prepared mixture (9:1, v:v) of methanol and concentrated sulphuric acid (H\(_2\)SO\(_4\)) in 20-ml glass centrifuge tubes while mixing gently by hand. The mixture was let to stand overnight in the dark.
at 4°C before centrifugation at 1500 rpm for 10 min to precipitate the protein. The supernatant was filtered through 2 sheets of Whatman No.1 filter paper in clean 50-ml centrifuge tubes. Chloroform (one tenth of the filtrate volume) was added to the filtrate and mixed vigorously before the addition of ice-cold twice distilled water (a volume equal to that of filtrate) and centrifugation at 2,000 rpm for 5 min. The lower phase, which contained the methylated pigments in chloroform, was collected. The extraction procedure with chloroform was repeated twice. The total chloroform extract was dried by filtration through cotton wool and anhydrous sodium sulphate (Na₂SO₄) in a Pasteur pipette and collected in a conical test tube. The chloroform was evaporated by gentle blowing with nitrogen and the pigments were redissolved in a few microlitres of fresh chloroform and then subjected to t.l.c.

5.2.4 T.l.c. of methylated haem and haem products

Chromatographic separation of the methylated haem and haem products was obtained on a silica-gel plate using methanol/chloroform (3:20) as the developing system. After the chromatographic run, pigments were eluted from the silica with 5 ml of methanol containing 0.2 ml concentrated hydrochloric acid (HCl). The methanol/HCl was then evaporated till a negative pH paper test was obtained and the dry pigments were dissolved in fresh chloroform.
for radioactivity determination in Beckman PM scintillant or applied
again to silica-gel plates for further purification by t.l.c. as
described above.

5.2.5 H.p.l.c. of non-methylated haem and haem products

In an attempt to separate the various unmethylated haem products
from haem, an h.p.l.c. method was set up using a reversed phase
column (uBondapak C₁₈, Waters, 300 x 3.9 mm internal diameter) and
an ion-pairing gradient elution system. Two volumes of 80% methanol
in water containing 2.5 mM TBA were added to the mixture containing
the haem products to be separated and mixed by whirling. After
centrifugation at high speed in an Eppendorf bench centrifuge for 2
min to precipitate any suspended particles, 20 ul of the supernatant
were injected onto the column and eluted using the following
solvents: A) 35% methanol in water (twice distilled, deionized and
gas-free) containing 2.5 mM TBA, B) 95% methanol containing 1 mM
TBA. Elution was for 1 min with 100% A, for 13 min with a linear
gradient to 58.3% A in the A + B mixture and, for the subsequent 7
min, with a steeper linear gradient to 100% B. This concentration
was then maintained for the last 14 min before adjustment of the
system to starting conditions (100% A). The chromatographic
conditions are reported in Table 14 and the gradient is represented
in Fig. 25.
Table 14. Chromatographic conditions of the h.p.l.c. method for detection of haem and haem products

| Apparatus: | Pump A: Altex, model 100 A  
Pump B: Altex, model 110 A |
| Column: | Waters, uBondapak C\textsubscript{18} 10 \textmu m particles, 3.9 x 300 mm |
| Guard column: | Whatman, C\textsubscript{18} CO:PELL ODS 30-38 \textmu m particles, 3.9 x 30 mm |
| Injector: | Altex, 20 ul |
| Detectors: | absorbance: E400, Cecil CE 2012 spectrophotometer  
fluorescence: Gilson filter fluorimeter |
| Integrator: | Shimadzu model C-R1A |
| Solvent: | methanol in water, containing 2.5 mM tetrabutyl-ammonium hydroxide in phosphate buffer, pH 7.5 |
| Gradient: | 35-95% methanol with curve as in Fig. 25 |
| Flow rate: | 1 ml/min |
| Column temp.: | 21-23\textdegree C |
Fig. 25. Gradient of the h.p.l.c. method for detection of haem and haem products. See text and Table 14 for details.
After measuring the absorbance at 400 nm in the eluate, the eluate was collected in 0.5- or 1-min fractions and the radioactivity in each of these fractions was determined after addition of 4 ml MP scintillant by scintillation.

5.3 RESULTS

5.3.1 Suicidal CCl₄-dependent destruction of [¹⁴C]-haem

It should be noted that the pattern of labelling of haem is different when either [4-¹⁴C]-ALA or [5-¹⁴C]-ALA is used as a precursor, since with [4-¹⁴C]-ALA the labelling is entirely in the pyrrole rings, whereas with [5-¹⁴C]-ALA the four methene bridges of haem are also labelled (Fig. 24). This means that any carbon monoxide formed from the methene bridge carbon atoms of haem, as for instance, during physiological haem degradation by haem oxygenase, would be radioactive when [5-¹⁴C]-ALA but not [4-¹⁴C]-ALA is used.

When MHA containing [¹⁴C]-haem from [5-¹⁴C]-ALA was incubated under the usual anaerobic conditions without any addition or with CCl₄/sodium dithionite, as in the experiment of Fig. 26, there was no loss of radioactivity in either case. The recovery of radioactivity was 102 and 105% in control and CCl₄/dithionite incubation, respectively, indicating that radioactive volatile products of haem, and particularly CO, were not formed. When, after
incubation, the radioactivity was extracted in methanol/TBA and the extracts were injected into the h.p.l.c. system and eluted in 0.5-min fractions (as described in 5.2.5) both the absorbance at 400 nm and the radioactivity associated with the haem peak markedly decreased after incubation with CCl₄/dithionite (Fig. 26).

Whereas the radioactivity lost from the haem peak was almost completely recovered in several new fractions eluted immediately before and after haem, the retention of the 400 nm absorbance in these new peaks was only negligible. This suggested conversion of haem into products which retained quantitatively the original haem radioactivity but which had largely lost the absorbance at 400 nm.

Preliminary experiments involving injection and chromatography of several porphyrin standard solutions showed that the h.p.l.c. technique could separate a wide range of porphyrins, from uroporphyrin to mesoporphyrin (data not shown). So if any modified porphyrin were to be formed during CCl₄-dependent haem loss, the h.p.l.c. fluorescence detector would have shown a new peak corresponding to the modified porphyrin. Since, however, no peak of fluorescence was seen throughout the chromatogram (results not shown) the interpretation was that no significant amounts of known, naturally occurring porphyrins were produced as a result of haem degradation. This confirmed previous results (see 3.3.3.1).
Fig. 26. Absorbance at 400 nm (A) and recovery of radioactivity (R) in the h.p.l.c. eluate after anaerobic incubation of [14C]-haem in the absence and presence (..., — — --- in A and △—△, ▲—▲ in R, respectively) of CCl₄/dithionite. Both incubations were at 25°C for 5 min in rubber-stoppered minivials and contained [14C]-haem (1.25 mM, 97 nCi/μmol, from [5-14C]-ALA) in 60 μl of 0.1 M Na₂HPO₄ buffer, pH 7.4 in the presence of the usual oxygen scavenging system. At time 0, sodium dithionite and CCl₄ were added to one incubation mixture to obtain a final concentration of 2 mM for both compounds. At the end of the incubation the haem and haem-derived pigments were extracted and injected into the h.p.l.c. system. After detection of the absorbance and fluorescence (not shown) in the eluate, samples were collected in 0.5 min fractions and counted.
5.3.2 Recovery of radioactivity from $^{14}$C-haem after h.p.l.c.

The recovery of radioactivity after incubation of $[^{14}$C]-haem with CCl$_4$/dithionite, extraction and injection of the resulting pigments into the h.p.l.c. system is reported in Table 15.

Recovery of radioactivity in the methanol/TBA mixture was quantitative for both control and test incubation (103 and 102% of the amount added as haem at the start of the incubation, respectively). In this experiment total radioactivity injected into the h.p.l.c. system was 1,922 and 1,908 dpm for control and test sample, respectively. Total recovery of injected radioactivity was similar for control and CCl$_4$/dithionite incubations, and was in excess of 90%.

Several radioactive products were found in the h.p.l.c. fractions eluting both before and after haem. These fractions were called "non-haem fractions" since from preliminary experiments they were known to contain less than 5% of the total radioactivity eluting after injection of authentic $[^{14}$C]-haem into the h.p.l.c. system. These fractions also showed little absorbance at 400 nm and no fluorescence, but contained 43% of the original haem radioactivity. Thus, as a result of the incubation with CCl$_4$/dithionite, there was no change in the total radioactivity recovered after h.p.l.c. but the radioactivity appeared in several new fractions at the expense
Table 15. Recovery of radioactivity in haem and non-haem h.p.l.c. fractions after anaerobic incubation of [\(^{14}\text{C}\)]-haem in presence or absence (Control) of CCl\(_4\) and sodium dithionite.

<table>
<thead>
<tr>
<th>Fraction (No.)</th>
<th>Control dpm (% of total radioactivity injected)</th>
<th>CCl(_4)/sodium dithionite dpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>haem (41-48)</td>
<td>1,663 (87)</td>
<td>949 (50)</td>
</tr>
<tr>
<td>non-haem (1-40, 49-98)</td>
<td>144 (7)</td>
<td>816 (43)</td>
</tr>
<tr>
<td>all fractions (1-98)</td>
<td>1,806 (94)</td>
<td>1,765 (93)</td>
</tr>
</tbody>
</table>

Experimental conditions were as for the experiment of Fig. 26.
of haem radioactivity. The small portion of the initial radioactivity recovered in the "non-haem fractions" of the control incubation (7%) was attributed to initial and final elution of the haem peak and not to new radioactive products.

5.3.3 Suicidal inactivation of haem by CCl₄ and the binding of CCl₄ metabolites to haem

In order to investigate whether any metabolite of CCl₄ was bound to haem products, [¹⁴C]-CCl₄ and unlabelled haem (as MHA) were incubated anaerobically together with sodium dithionite, and the products of haem degradation so obtained were extracted into methanol/TBA, as described in 5.2.5, and injected into the same h.p.l.c. system used in the experiments with [¹⁴C]-haem.

Almost no radioactivity was recovered from [¹⁴C]-CCl₄ incubated with MHA alone (Fig. 27, A) or with dithionite alone (results not shown). In contrast, when [¹⁴C]-CCl₄ was incubated with MHA and dithionite together, two major areas of radioactivity were detected; a sharp peak eluting approximately 5 min after injection and a broad area of radioactivity eluting between 15 and 28 min. The first is unlikely to represent radioactivity bound to haem as no peak of radioactivity was seen in the same fractions when [¹⁴C]-haem and unlabelled CCl₄ were incubated with dithionite under similar experimental conditions (Fig. 27, B). Approximately 20% of the
Fig. 27. Recovery of radioactivity in h.p.l.c. fractions containing haem products formed during sodium dithionite-reduced incubation of either $[^{14}C]$-CCl$_4$ with unlabelled haem (A) or unlabelled CCl$_4$ with $[^{14}C]$-haem (B). Experimental conditions in A were those of experiment 2 of Table 16. After a 10 min incubation 50 ul of each incubation mixture were added to 100 ul methanol/TBA, mixed and then 20 ul of this mixture were injected into the h.p.l.c. system. The radioactivity eluted up to 35 min as described in 5.2.5 was then measured in each one min fraction. In presence of dithionite (A, ▲) approximately 85% of the radioactivity injected was recovered after h.p.l.c., whereas in its absence (A, △) the recovery was almost negligible (< 5%).

In B, both incubations were for 15 min at 25°C and contained 75 nmol $[^{14}C]$-haem (0.097 uCi/umol, from [5-$^{14}$C]-ALA) in 60 ul of the usual anaerobic buffer. At time 0, 0.12 umol CCl$_4$ and 0.12 umol sodium dithionite (1.9 mM approximate final concentration for both compounds) were added anaerobically to test (▲) but not to control (△) incubation. At the end of the incubation time, 25 ul of each incubation mixture were added to 50 ul methanol/TBA, mixed and then 20 ul of the mixture were injected into the h.p.l.c. system and eluted as described in 5.2.5, before measuring the radioactivity in each one-min fraction.
radioactivity injected was recovered in the second area of elution, corresponding to those fractions which were known to contain haem or haem products. This indicates that the activation of CCl₄ by haem resulted in the appearance of CCl₄ metabolites and in significant binding of some of these to haem. In the experiment of Fig. 27, the recovery of radioactivity injected as [¹⁴C]-haem after h.p.l.c. was 90 and 94 % in presence and absence of CCl₄/dithionite, respectively.

5.3.4 The stoichiometry between haem inactivation and binding of CCl₄ metabolites to haem products

In the attempt to compare the amount of CCl₄ binding to haem products with the extent of haem loss, [¹⁴C]-CCl₄ was incubated with unlabelled haem (as MHA) and sodium dithionite. When the CCl₄-dependent haem loss was measured as the difference between the area of the h.p.l.c. haem peak obtained in absence and that obtained in presence of [¹⁴C]-CCl₄, a 1:1 stoichiometry was calculated, in two separate experiments, between haem lost and adduct formed (Table 16).

These results strongly indicate that in this non-enzymic system one activated molecule of CCl₄ which interacts with the haem is sufficient to cause haem inactivation.
Table 16. Stoichiometric haem loss and \([^{14}\text{C}]-\text{CCl}_4\)-derived adduct formation

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Haem loss (nmol)</th>
<th>Radioactivity from ([^{14}\text{C}]-\text{CCl}_4) recovered bound to haem products (nmol CCl(_4) equivalent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.12</td>
<td>0.12</td>
</tr>
<tr>
<td>2</td>
<td>0.23</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Incubations were at 22°C for 10 min and contained 20.3 nmol haem, as MHA, and sodium dithionite (1.9 mM approximate final concentration) in 0.2 ml of the usual anaerobic buffer. At time 0, 20.8 (Expt. 1) or 52.0 (Expt. 2) nmol \([^{14}\text{C}]-\text{CCl}_4\) (4.0 uCi/umol) in methanol was added to the incubation mixture to start the reaction. The control incubation contained MHA and sodium dithionite but no \([^{14}\text{C}]-\text{CCl}_4\). At the end of the incubation, 50 ul of the incubation mixture were added to 100 ul of the methanol/TBA solution before injecting 20 ul of this mixture into the h.p.l.c. system. The haem present in the eluate was quantitated by measuring the absorbance at 400 nm as total integrated area under the h.p.l.c. haem peak (after a preliminary calibration with known amounts of \([^{14}\text{C}]-\text{haem}\)), assuming the contribution in absorbance of the haem-derived products to be negligible. Then the radioactivity was measured in each fraction as described in 5.2.5. The haem-bound radiolabel was calculated from the radioactivity recovered in those fractions known to contain haem and haem-derived pigments and was converted into CCl\(_4\) equivalents knowing the specific activity of \([^{14}\text{C}]-\text{CCl}_4\). The CCl\(_4\)-dependent loss of haem was calculated as the difference between the loss due to CCl\(_4\)/dithionite and that due to dithionite alone, measured in the control incubation (data not shown).
5.3.5 Reductive activation of \( \text{CCl}_4 \) to non-volatile, water soluble products by haem in the form of methaemalbumin

The mechanism of \( \text{CCl}_4 \) reductive activation by haem was further investigated by following the fate of the radiolabel after incubation of \( \text{[}^{14}\text{C}]\text{-CCl}_4 \) with haem and/or sodium dithionite. Radioactivity was measured in the fractions obtained by extraction of the incubation mixtures with \( n \)-hexane and subsequent treatment of the aqueous phase with trichloroacetic acid (Table 17).

The radiolabel was fully recovered only in the incubation containing both haem and sodium dithionite (incubation 3), whereas it was largely lost when either dithionite or haem were omitted (incubation 1 and 2, respectively). Moreover, the bulk of the radioactivity was extractable by \( n \)-hexane in incubations 1 and 2, whereas, when haem and dithionite were present together, most radioactivity was recovered in the aqueous phase, either in solution or bound to the precipitated albumin.

5.3.6 T.l.c. of haem degradation products

The haem degradation products and the residual haem present in the incubation mixture at the end of an anaerobic incubation of MHA with \( \text{CCl}_4 \)/dithionite were investigated further by t.l.c. The pigments were methylated in methanol/\( \text{H}_2\text{SO}_4 \) as described under
Table 17. Distribution of radioactivity in different fractions obtained after anaerobic incubation of $[^{14}\text{C}]-\text{CCL}_4$ with methaemalbumin (MHA) and/or sodium dithionite

<table>
<thead>
<tr>
<th>Fraction</th>
<th>MHA (1)</th>
<th>Dithionite (2)</th>
<th>MHA + Dithionite (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total final incubation mixture</td>
<td>23,370 (25.9)</td>
<td>27,620 (30.6)</td>
<td>94,870 (105.2)</td>
</tr>
<tr>
<td>n-hexane extract</td>
<td>18,060 (20.0)</td>
<td>19,620 (21.8)</td>
<td>3,320 (3.7)</td>
</tr>
<tr>
<td>supernatant</td>
<td>780 (0.9)</td>
<td>3,750 (4.2)</td>
<td>51,300 (56.8)</td>
</tr>
<tr>
<td>pellet</td>
<td>496 (0.6)</td>
<td>- * ( - )</td>
<td>15,060 (16.7)</td>
</tr>
</tbody>
</table>

Radioactivity lost during:

<table>
<thead>
<tr>
<th>Incubation</th>
<th>MHA (1)</th>
<th>Dithionite (2)</th>
<th>MHA + Dithionite (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation</td>
<td>66,776 (74.0)</td>
<td>62,526 (69.4)</td>
<td>nil</td>
</tr>
<tr>
<td>Fractionation</td>
<td>4,034 (4.5)</td>
<td>4,250 (4.7)</td>
<td>25,190 (27.9)</td>
</tr>
</tbody>
</table>

Incubations were for 10 min at 22° C in rubber-stoppered glass minivials containing 200 µl 0.05 M Na$_2$HPO$_4$ buffer, pH 7.4, in presence of the oxygen-scavenging system described in 2.2.7. Incubations (1) and (3) contained 226 nmol haem (as MHA) while incubation (2) contained no haem. Sodium dithionite (approx. 400 nmol) was added to incubations 2 and 3 but not to incubation 1, just before the addition, at time 0, of 20.8 nmol of $[^{14}\text{C}]-\text{CCL}_4$ (4.0 uCi/µmol). At the end of the incubation, the radioactivity present in the incubation mixture was measured by taking 10 µl into 4 ml MP scintillant. One hundred µl of the remaining incubation mixture were then extracted twice with two volumes of n-hexane and the radioactivity present in the total extract was measured by taking 20 µl into the MP scintillant and counting. Finally, protein was precipitated by adding an equal volume of a 20% (v:v) aqueous solution of trichloroacetic acid to the water phase remaining after the extraction with n-hexane. After centrifugation, radioactivity was measured in supernatant and total pellet. *, No precipitate was formed from incubation 2, due to the absence of albumin in this sample.
Materials and methods. The methyl esters so obtained, together with those obtained from untreated and dithionite- or \( \text{CCl}_4 \)-treated controls, were applied to a silica gel plate for t.l.c. and were developed by a methanol/chloroform mixture (see 5.2.4). The recovery of radioactivity after anaerobic incubation of \(^{14}\text{C}]\)-haem (as MHA) with and without \( \text{CCl}_4 \) and/or sodium dithionite, and following methylation, extraction and t.l.c. of the resulting pigments are reported in Table 18.

The haem concentrations measured at the end of the incubations with \( \text{CCl}_4 \), sodium dithionite or both (\( \text{a} \)) were 98.7, 95.1 and 22.2 % of that found in control incubation, respectively. This was in good agreement with previous results (see 3.3.3.1). No significant loss of radioactivity was observed during any incubation, suggesting that no significant amounts of volatile haem products were formed. This was also in agreement with previous findings (see 5.3.1 and 5.3.2). On the contrary, the total loss of radioactivity after methylation and t.l.c. of pigments obtained in the incubation with \( \text{CCl}_4 \)/dithionite was approx. double (approx. 40 % of the initial radioactivity) of that found after the incubations where \( \text{CCl}_4 \) and/or dithionite were omitted (approx. 20 % of the initial radioactivity).

After methylation and t.l.c., authentic haem gave two bands, a main band \( (R_f = 0.67) \) and a much smaller band \( (R_f = 0.20) \), which probably corresponded to the dimethyl and monomethyl ester,
Table 18. Recovery of radioactivity after anaerobic incubation of [\(^{14}\text{C}\)]-haem with CCl\(_4\) and/or sodium dithionite

<table>
<thead>
<tr>
<th>Addition</th>
<th>Recovered at the end of incubation (A)</th>
<th>Recovered after methylation and t.l.c. (B)</th>
<th>Lost (A-B)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm (%)*</td>
<td>dpm (%)*</td>
<td>dpm (%)*</td>
</tr>
<tr>
<td>None (control)</td>
<td>26,880 (98.9)</td>
<td>21,522 (79.2)</td>
<td>5,358 (19.7)</td>
</tr>
<tr>
<td>CCl(_4)</td>
<td>26,933 (99.1)</td>
<td>20,618 (75.9)</td>
<td>6,315 (23.2)</td>
</tr>
<tr>
<td>dithionite</td>
<td>27,149 (99.9)</td>
<td>23,061 (84.8)</td>
<td>4,088 (15.1)</td>
</tr>
<tr>
<td>(\text{CCl}_4/\text{dithionite})</td>
<td>(\text{a } 27,202 (100.0))</td>
<td>15,968 (58.7)</td>
<td>11,234 (41.3)</td>
</tr>
<tr>
<td></td>
<td>(\text{b } 27,207 (101.2))</td>
<td>16,440 (61.2)</td>
<td>10,767 (40.0)</td>
</tr>
</tbody>
</table>

*, \% of radioactivity added to incubation; \(\dagger\), \(\text{a}\) and \(\text{b}\) represent duplicate runs. Incubations were at 21°C for 5 min and contained 0.5 umol \([\^{14}\text{C}]\text{-haem}\) (0.026 uCi/umol, from \([4-\^{14}\text{C}]\text{-ALA}\) (as MHA) and, where indicated, 4 umol CCl\(_4\) and/or 5.8 umol sodium dithionite in 2 ml of the anaerobic buffer. At time 0, the reaction was started by injecting into the incubation mixture either CCl\(_4\) or the sodium dithionite solution or both. No addition was made to the control incubation. At the end of the incubation, 100 ul of the mixture were pipetted into 2.4 ml pyridine/NaOH for haem measurement, 10 ul were added to 10 ml Beckman MP scintillant for counting radioactivity, and the approximately 1.9 ml of incubation mixture remaining were transferred dropwise into ice-cold methanol/H\(_2\)SO\(_4\) for methylation (see 5.2.3). The methylated pigments were subjected to t.l.c. (see Fig. 28) and eluted as indicated under Materials and methods, taken to dryness, dissolved in chloroform and added to 10 ml MP scintillant for determination of radioactivity.
respectively (Fig. 28). Identical results were obtained when MHA was incubated with CC\textsubscript{4} in the absence of dithionite; when dithionite was incubated with MHA in the absence of CC\textsubscript{4} the two bands described above were still observed but they were slightly less marked, and a new, faint fluorescence band was noted (R\textsubscript{f} = 0.82), possibly representing protoporphyrin IX produced from haem by dithionite-dependent loss of iron. Moreover two additional bands were observed with R\textsubscript{f} 0.12 and 0.07. A remarkably different picture was seen when both CC\textsubscript{4} and dithionite were present in the same incubation mixture. In this case several bands were observed and some deposition of pigment was seen all throughout the chromatographic run. The two methylated haem bands with R\textsubscript{f} = 0.67 and 0.20 were still observed, although they were far less intense than in the three previous control plates. The faint fluorescent product (R\textsubscript{f} = 0.82) and the two additional bands with R\textsubscript{f} 0.12 and 0.07 produced by dithionite were also seen and, in addition, two new bands fairly close to each other (R\textsubscript{f} = 0.83 and 0.77, respectively) were also observed (products A and B, respectively).

5.3.7 Partial characterization of CC\textsubscript{4}-dependent haem products

An attempt was made to isolate and characterize products A and B. These were isolated and purified twice by t.l.c. as described in
Fig. 28. T.l.c. of the methyl esters of haem and haem products after anaerobic incubation of $[^{14}\text{C}]$-haem (as MHA) without any addition (control), or with CCl$_4$ and/or sodium dithionite. Incubations were those of the experiment of Table 18. After incubation and methylation as described in 5.2.3 the methylated pigments were subjected to t.l.c. on a silica-gel plate as described in 5.2.4.
5.2.4. The t.l.c. picture of the CCl₄-dependent haem products after methylation, isolation and purification by t.l.c. is reported in Fig. 29, together with that of authentic and residual haem.

Products A and B were then eluted from silica as described in 5.2.4 and partially characterized by their absolute reduced (Fig. 30) and difference (reduced against oxidised) spectra in pyridine/NaOH (Table 19). When compared to those of authentic haem the spectra of products A and B show extinction coefficients which are decreased to about 20%. Slightly lower values were also calculated for the extinction coefficients of residual haem when compared with authentic haem, possibly due to some contamination with product B (see also Fig. 30, RH). Products A and B showed haem-like spectra with an hypsochromic shift of Soret, α and β peaks. On the contrary an extensive investigation using a wide range of excitation and emission wavelengths showed products A and B to be non-fluorescent when examined either by the methanol/perchloric acid method or by the oxalic acid technique (see 3.2.4). These results indicate that products A and B may be haem-like compounds with the iron still present. From the amount of radioactivity recovered in products A and B after purification, and assuming that they both have the same specific radioactivity as the original [¹⁴C]-haem, the two products of haem degradation were quantitated and their extinction coefficients tentatively calculated (Table 19).
Fig. 29. T.l.c. profile of authentic haem (AH) and isolated/purified residual haem (RH) and products A, B and, possibly, C. The incubation was at 21°C for 5 min. and contained 265 nmol [14C]-haem (0.026 uCi/umol, from [4-14C]-ALA) as MHA, 4 umol CCl4 and 5.8 umol sodium dithionite in 2 ml of the anaerobic buffer. At the end of the incubation 50 ul of the incubation mixture were pipetted into pyridine/NaOH for the haem assay and 20 ul were used for counting the radioactivity. The incubation remaining was transferred into methanol-H2SO4 for methylation as described in 5.2.3. After extraction and t.l.c. of the methylated pigments as for Fig. 28, the several bands obtained ("A", "B", "C" and "RH") were separately eluted from silica with methanol-HCl and applied again, together with authentic methylated haem (AH) to a silica-gel plate for purification as described in 5.2.4.
Fig. 30. Reduced absolute spectra in pyridine/NaOH of products A and B and residual (RH) or authentic (AH) haem. Experimental conditions were those of Fig. 29. See legend to Table 19 for details.
Table 19. Absorption spectra in pyridine/NaOH of the dimethyl esters of authentic or residual [\(^{14}\text{C}\)]-haem and products A and B

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Soret (\lambda_{\text{max}}) (\text{nm})</th>
<th>(\lambda_{\text{min}}) (\text{nm})</th>
<th>(\alpha_{\text{max}}) (\text{emM})</th>
<th>Reduced minus oxidised</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute reduced</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control haem</td>
<td>418</td>
<td>524</td>
<td>539</td>
<td>555</td>
</tr>
<tr>
<td></td>
<td>(147.8)</td>
<td>(20.2)</td>
<td>(117.2)</td>
<td>(19.4)</td>
</tr>
<tr>
<td>residual haem</td>
<td>417</td>
<td>523</td>
<td>538</td>
<td>554</td>
</tr>
<tr>
<td></td>
<td>(133.0)</td>
<td>(15.8)</td>
<td>(94.7)</td>
<td>(15.1)</td>
</tr>
<tr>
<td>product A</td>
<td>392</td>
<td>520</td>
<td>536</td>
<td>550</td>
</tr>
<tr>
<td></td>
<td>(*)</td>
<td>(3.1)</td>
<td>(*)</td>
<td>(3.1)</td>
</tr>
<tr>
<td>product B</td>
<td>392</td>
<td>520</td>
<td>536</td>
<td>551</td>
</tr>
<tr>
<td></td>
<td>(*)</td>
<td>(5.5)</td>
<td>(*)</td>
<td>(4.7)</td>
</tr>
</tbody>
</table>

\(\text{emM} = \text{emM}^{-1}\cdot\text{cm}^{-1}\); \(\text{emM}_{\alpha_{\text{min}}} = \text{emM}^{-1}\cdot\text{cm}^{-1}\) \(\alpha_{\text{min}}\);*, not calculated due to high dithionite absorbance at this wavelength. Authentic or residual haem and products A and B were methylated and purified as described in 5.2.3 and 5.2.4, respectively. The dimethyl esters so obtained were dissolved in 2 ml of pyridine before the addition of 3.6 ml of 0.3 N NaOH. The mixture was then equally divided into two 3-ml quartz cuvettes stoppered by a teflon cap. A few milligrams of solid sodium dithionite were added to the test cuvette and, in the case of the difference spectra, potassium ferricyanide was added to the reference cuvette to a 75 \u03bcM final concentration. The absolute reduced and the difference (reduced minus oxidised) spectra were then recorded between 600 and 370 nm. The \(\text{emM}^{-1}\cdot\text{cm}^{-1}\) given were calculated from the specific radioactivity of the haem added at the start of the incubation, assuming the specific radioactivity of haem products and that of haem to be identical.
5.4 DISCUSSION

The results presented in this section of the thesis provide additional and, possibly, conclusive evidence that under anaerobic conditions CCl₄ can be activated reductively by haem compounds in solution in the absence of the cytochrome P-450 apoprotein. This view is supported, for instance, by the results of Table 17 indicating that under reducing conditions haem catalysed the transformation of CCl₄ into less volatile, more water-soluble products. This non-enzymatic activation was, however, accompanied by irreversible degradation of haem to several unidentified products, as shown by both h.p.l.c. and t.l.c. techniques. The loss of absorbance at 400 nm observed after incubation of [¹⁴C]-haem with CCl₄ and dithionite and subsequent analysis of the resulting radioactive products by h.p.l.c. is in agreement with the previous observation of a dramatic loss of the pyridine-haemochrome reaction under similar conditions. The loss of absorbance was not due to low recovery of the haem compounds after incubation since no significant haem-derived radioactivity was lost in this experiment.

As previously mentioned carbon monoxide is a well known and specific product of the physiological breakdown of haem by haem oxygenase which involves scission of the porphyrin ring at the α-methene bridge and conversion of the bridge carbon into CO. It was
also emphasized that $^{14}$C-haem from [5-$^{14}$C]-ALA, but not from [4-$^{14}$C]-ALA, is labelled at the bridge methene carbon atoms (see Fig. 24). So, $^{14}$C-haem from [5-$^{14}$C]-ALA allowed the investigation of the possible formation of haem-derived CO during the anaerobic activation of CCl$_4$ by microsomal cytochrome P-450. The quantitative recovery of radioactivity after incubation in this experiment strongly suggested that volatile haem products such as CO were not formed. This confirms that CO is in fact produced during reductive activation of CCl$_4$ by cytochrome P-450 or haem alone, as described elsewhere in the present thesis, but only as a metabolite of CCl$_4$.

It has been proposed (Guzelian & Swisher, 1979) that the in vitro and in vivo degradation of cytochrome P-450 haem by CCl$_4$ is similar in part to that caused by 2-allyl-2-isopropylacetamide (AIA), a well known suicide substrate of cytochrome P-450 (Loosemore et al., 1981; Ortiz de Montellano & Mico, 1981), in that in both cases the loss is promoted by a non-CO-forming mechanism and is independent on lipid peroxidation. Radiolabelling experiments showed (Ortiz de Montellano et al., 1978) that the abnormal "green pigments" produced by AIA are 1:1 covalent adducts of AIA with protoporphyrin IX, the iron-free precursor of haem. A 1:1 stoichiometry between haem lost and reactive metabolites bound to haem products was also observed with CCl$_4$ in the present study using a non-enzymic model of CCl$_4$ activation. This observation provided
circumstantial evidence in favour of the hypothesis put forward by de Groot & Haas (1980) that covalent binding of CCl\textsubscript{4} reactive metabolites to the prosthetic haem moiety is the cause of cytochrome P-450 loss.

The products of haem degradation, however, appear to be different with the two compounds. With AIA cytochrome P-450 haem is converted into N-alkylated porphyrins (De Matteis, 1971) whereas with CCl\textsubscript{4}, modified porphyrins were apparently not formed and the haem appears from h.p.l.c. analysis to be transformed into several unidentified products.

Using a non-enzymic model containing [\textsuperscript{14}C]-haem from [4-\textsuperscript{14}C]-ALA, two radioactive, non-fluorescent, non-volatile haem-derived pigments have been isolated and they have been partially characterized by t.l.c. after methylation. These products showed a haem-like UV-visible spectrum in pyridine/NaOH solution, with an hypsochromic shift of the Soret and of the $\alpha$ and $\beta$ maxima as compared with authentic haem. A significantly lower $\epsilon$M$^{-1}$cm$^{-1}$ was calculated under these conditions for both products when compared to haem. These pigments did not show any fluorescence under UV light, nor did they become fluorescent when heated in saturated oxalic acid for 30 min, indicating that under these conditions they no longer maintained a simple tetrapyrrolic ring. This was in agreement with the little absorbance at 400 nm and no fluorescence shown in the
h.p.l.c. elution profile by the haem products formed during the incubation of $^{14}$C-haem with CCl$_4$/dithionite (Fig. 26). The possibility that pigments A and B are labile haem-like compounds with a CCl$_4$ metabolite complexed on to the central iron so that pyridine cannot complex to form a pyridine haemochrome and strong acidic treatment with oxalic acid can lead to irreversible modification of the tetapyrrolic structure, although much less likely, cannot be ruled out. These aspects require further work.
6. General Discussion

The present project was undertaken to investigate the molecular mechanism of, and the reactive species responsible for, the inactivation of cytochrome P-450 during the reductive metabolism of CCl₄.

In this study, results obtained from several experimental approaches and using different techniques have shown conclusively that haem is the critical, suicidal target of the reductive activation of CCl₄. Early experiments indicated that anaerobic incubation of microsomes with CCl₄ in presence of NADPH or sodium dithionite resulted in irreversible loss of both cytochrome P-450 and its prosthetic group, haem (Fig. 14). No significant loss was observed with NADPH, in the absence of CCl₄. With dithionite alone some loss was noted in some of the experiments, but this was small (usually 5-10% of total loss measured) and somewhat variable, perhaps due to residual traces of oxygen, resulting in transient reactive species with dithionite.

These findings confirm previous reports (de Groot & Haas, 1980) and support the hypothesis (see 1.5) that inactivation of the prosthetic haem by reactive metabolites of CCl₄ is the causal event in the loss of the haemoprotein. In fact, although the loss of cytochrome P-450 during metabolic activation of CCl₄ has been
recognised for some time (Sasame et al., 1968), the molecular mechanism and the reactive species involved have not been fully clarified. Initially, the loss of the haemoprotein had been attributed to CCl$_4$-induced peroxidative degradation of lipids in hepatocyte membranes, a free radical chain reaction process thought to be started by ·CCl$_3$, (Glende et al., 1976; Masuda & Murano, 1977). The observation, however, that in microsomes or isolated hepatocytes the free radical-scavenger promethazine significantly prevented CCl$_4$-stimulated lipid peroxidation, but had no protective effect against CCl$_4$-dependent destruction of cytochrome P-450, strongly suggested that the damaging effect of CCl$_4$ on the cytochrome did not involve lipid peroxidation (Poli et al., 1981). It was concluded by these authors that metabolic products of CCl$_4$ could damage microsomal components in various ways: lipid peroxidation, covalent binding and direct attack of reactive intermediates on cytochrome P-450.

A possible role for lipid peroxidation in CCl$_4$-dependent cytochrome P-450 destruction in vitro was even less likely following the finding that even under strictly anaerobic conditions, activation of CCl$_4$ caused an important loss of the haemoprotein (Yamazoe et al., 1979; de Groot & Haas, 1980; Masuda, 1981). de Groot et al. (1981) showed that under anaerobic conditions the loss of the cytochrome was accompanied by loss of the prosthetic haem, as
as a result, they suggest, of the binding of CCl$_4$ reactive metabolites, ·CCl$_3$ or :CCl$_2$, to haem. The covalent binding of CCl$_4$ metabolites to the haem moiety of cytochrome P-450 during anaerobic incubation of CCl$_4$ with NADPH-supplemented rat liver microsomes was reported by Fernandez et al. (1982). These authors also obtained labelled haem and haem-breakdown products from the liver of rats treated with $^{14}$C]-CCl$_4$ at dosages known to produce a significant loss of cytochrome P-450. They observed also that covalent binding of CCl$_4$ to proteins from CO-binding particles was higher than that to whole microsomal protein and concluded that the covalent binding of CCl$_4$ reactive metabolites to the haem and protein moiety of cytochrome P-450 might be involved in the process of destruction of the haemoprotein.

The results of the present study support the view that binding of CCl$_4$ reactive metabolites to the prosthetic haem, not the apoprotein is primarily responsible for the inactivation of the haemoprotein.

Consistent results in this direction were obtained using three different, strictly anaerobic systems: (i) rat liver microsomal fractions (Fig. 14, Table 3), (ii) a chemical system with haem complexed to albumin in solution (Table 7) and (iii) a "mixed" system in which the haem-albumin complex was added to the microsomal suspension (Table 10). In all cases addition of CCl$_4$ and a suitable electron donor to strictly maintained anaerobic incubations resulted
in the loss of the haem catalyst.

Particularly interesting was the observation - obtained in the chemical system - that haem alone, in the absence of the apoprotein of cytochrome P-450, could activate $\text{CCl}_4$ to haem-destroying metabolites. Several haem compounds, from protohaem to mesohaem and deuterohaem, were tested and they were all found to be able to catalyze the reaction. The observations that i) protoporphyrin IX, the iron-free physiological precursor of haem, did not catalyze, nor was it a secondary target in the reaction (Table 9) and that ii) high concentrations of CO - a selective ligand of reduced haem - afforded a significant protection against haem loss (Table 8) suggest that the activation of $\text{CCl}_4$ by haem is a typical "suicidal" reaction which requires a reduced haem iron with available axial ligand positions. It should be noted that the chemical reaction was much faster than the enzymatic reaction catalysed by microsomes.

Based on these results and the additional observation that the anaesthetic halothane was also able to promote suicidal haem degradation (Table 12), a general reaction for the suicidal reductive activation of polyhalogenated alkanes by haem can be postulated. This reaction (Fig. 31) may involve the following sequential steps: 1. chemical or enzymatic reduction of haem; 2. interaction of reduced haem with the polyhalogenated alkane; 3. reductive dehalogenation of the alkane to a free radical; 4. binding
of the free radical to haem and, finally, 5. irreversible modification of the haem structure.

Steps 1., 2., 4. and 5. have been documented here, whereas the production of the free radical (step 3.) has not been specifically investigated in the present work. The covalent binding of haem, however, to \( \cdot \text{CCl}_3 \) produced from \( \text{CCl}_4 \) in a chemical reaction catalysed by benzoyl peroxide has been reported by Fernandez et al. (1982). The evidence supporting steps 1., 2., 4. and 5. can be summarized as follows. Both chemical (sodium dithionite) and enzymatic (microsomes or NADPH/NADPH-cytochrome P-450 reductase) reductants were found capable of reducing haem (step 1.) in solution, as shown unequivocally by the formation of a CO-haem difference spectrum (Fig. 19 and 20). The interaction of haem with the substrate or its metabolites (step 2.) was demonstrated by the formation of a reduced difference spectrum on addition of either \( \text{CCl}_4 \) or halothane to anaerobic, sodium dithionite-reduced methaemalbumin incubations (Fig. 8). In step 3 two reactions are actually represented: the reductive dehalogenation of the substrate by reduced haem and the subsequent re-reduction of the haem iron. The covalent binding of \( \text{CCl}_4 \) reactive metabolites to haem (step 4.) was established quantitatively using \([^{14}\text{C}]-\text{CCl}_4\) and measuring the \( \text{CCl}_4 \)-derived radioactivity bound to the products of haem degradation (Fig. 27 and Table 16).
Fe$^{+++}$ $\xrightarrow{1}$ Fe$^{++}$ $\xrightarrow{2}$ [Fe$^{++}$--RX] $\xrightarrow{3}$ Fe$^{++}$ + R· + X$^-$

$\xrightarrow{4}$ [Fe$^{++}$--R·] $\xrightarrow{5}$ Haem loss

Fe = haem
RX = carbon tetrachloride or halothane

Fig. 31. The suicidal, reductive activation of a polyhalogenated compound by haem. See text for explanation.
Calculation of the stoichiometry of the reaction showed that one molecule of CCl₄ binds one molecule of haem (Table 16). Finally, irreversible modification of the haem molecule (step 5.) was proved convincingly using spectrophotometric, fluorimetric and chromatographic techniques.

The non-enzymatic model system described here mimics in several respects the "suicidal" loss of haem occurring during cytochrome P-450-dependent, microsomal activation of CCl₄. Firstly in both systems the CCl₄-dependent destruction of haem, as measured by loss of the pyridine/haemochrome reaction, was accompanied by a stoichiometric loss of porphyrin fluorescence (Table 3 and 8), indicating that in both cases the haem moiety underwent an irreversible modification of the tetrapyrrolic system or some chemical change making it unable to survive the oxalic acid treatment or both. Secondly, in neither system did haem inactivation by CCl₄ result in the formation of known haem degradation products such as the "green pigments" formed from the breakdown of cytochrome P-450 haem by 2-allyl-2-isopropylacetamide (De Matteis, 1971), nor were fluorescent or volatile haem products formed. These results are in agreement with those of Guzelian & Swisher (1979) and indicate that the mechanism of haem modification by CCl₄ is different from that of 2-AIA and that of physiological haem degradation.
Thirdly, both with microsomes (Fernandez et al., 1982) and the present non-enzymic system, reactive metabolites of CCl₄, probably ·CCl₃, are produced which bind covalently to haem. Finally, the chemical reductant sodium dithionite and NADPH were able to support the reductive activation of CCl₄ by liver microsomes whereas sodium dithionite and microsomal or purified NADPH-cytochrome P-450 reductase, the latter in conjunction with NADPH, were able to catalyze the haem-dependent reaction (see 3.3.3.3 and 3.3.3.4). It would appear that both chemical and enzymatic transfer of electrons to haem can support the reaction in both systems.

As might be expected for enzyme inactivation reactions by "suicide" substrates, one molecule of CCl₄ would be likely to attack the same haem molecule responsible for its activation. The finding of a 1:1 stoichiometry between haem lost and CCl₄-derived metabolite bound to haem products observed with the chemical system (Table 16) is consistent with this expectation and suggests that a similar molecular mechanism may underlie both enzymatic and chemical activation of CCl₄.

Iron-porphyrin complexes have been extensively used as models for the catalytic site of cytochrome P-450 in order to study the metabolism of various polyhalogenated compounds (Mansuy, 1980) and in particular the molecular mechanism involved in CCl₄ activation (Mansuy et al., 1980). A haem model based on a biphasic system -
containing a partially reduced iron-mesotetraphenylporphyrin complex in the organic phase and sodium ascorbate in the water phase as the reducing agent - has been successfully used to mimic the reductive metabolism of CCl₄ by cytochrome P-450 (Mansuy, 1980). In order to validate further synthetic iron-porphyrin complexes as models for the cytochrome P-450 active site, it would be interesting to know whether any CCl₄-dependent loss of haem occurs also in this purely chemical, biphasic system. If this were not the case, i.e. no loss of haem were observed, the synthetic iron-porphyrin complexes may not be good models to mimic or investigate the suicidal, cytochrome P-450-dependent activation of CCl₄.

The present results have shown that haem alone is able to catalyze the "suicidal" non-enzymatic activation of CCl₄ or halothane in vitro under reductive conditions. A similar mechanism of activation leading in part to "suicidal" inactivation of the haem catalyst, but also in part to production of reactive derivatives available for damage of other targets in the cell, may be expected to occur in vivo in mitochondria or other haem-containing organelles. The loss of mitochondrial haem observed during incubation of rat liver mitochondria with halothane (Table 12) is consistent with this possibility. One can speculate, therefore, that any pool of haem, both "free" haem and haemoprotein haem, which is susceptible to reduction and which possesses at least one axial
ligand position free and accessible to CCl₄, may in theory catalyze CCl₄ activation in the cell and contribute significantly to the toxicity of the compound. This is consistent with the recent findings (Tomasi et al., 1987) that under reduced conditions mitochondria can activate CCl₄ in vitro.

It has been proposed (de Groot & Haas, 1981) that activation of CCl₄ to cytochrome P-450-damaging metabolites occurs at the cytochrome P-450 locus and requires interaction of the substrate with the haem iron. The protective effects of CO - a compound known to bind the reduced haem of cytochrome P-450 - against the loss of haem and cytochrome P-450 found in the present studies are in agreement with this proposal.

An important role of NADPH-cytochrome P-450 reductase in the activation of CCl₄ has been also recognized for some time (Slater & Sawyer, 1971b). The role of the flavoprotein is thought to be its ability to provide cytochrome P-450 with the electron equivalents necessary to reduce CCl₄. The flavoprotein, however, in presence of NADPH and in absence of oxygen can also reduce haem in solution directly, in the absence of the apoprotein, as shown in the present study (Fig. 20). This reduction was much slower than that occurring with dithionite as the reducing agent, but still it was fast enough to support CCl₄-dependent suicidal haem degradation (Fig. 21). These results indicate that under anaerobic conditions NADPH-cytochrome P-
450 reductase can activate CCl₄ through a mechanism which does not require cytochrome P-450. These findings may also provide an explanation for the unexpected loss of exogenous haem observed on addition of CCl₄ and NADPH to anaerobic incubations containing protohaem- or mesohaem-supplemented microsomes (Table 10 and Fig. 18, respectively). It is possible that electron equivalents from NADPH are transferred by the flavoprotein directly to methaemalbumin haem, without involvement of cytochrome P-450. This could be investigated by repeating these experiments using boiled microsomes or microsomal suspensions containing otherwise inactivated cytochrome P-450. If electron transfer from NADPH to haem in solution occurs through an active cytochrome P-450, no significant reduction or, in the presence of CCl₄, loss of proto- or mesohaem would be expected in these experiments. If, on the other hand, an active haemoprotein is not necessary, reduction/loss of haem would still be observed.

Both ·CCl₃ and :CCl₂ are reactive species reportedly capable of attacking the apoprotein and the prosthetic group of cytochrome P-450 (Noguchi et al., 1982a and b; Pohl & George, 1983; Fernandez et al., 1982). The inability of the carbene-trapping agent DMB to prevent the haem loss (Table 5) cannot be explained by postulating a limited access of the compound to :CCl₂, since identical concentrations of DMB, under the same conditions, significantly
inhibited the \( {\text{CCL}}_2\)-P-450 spectrum (Fig. 6). In preliminary experiments DMB gave a typical type I binding spectrum with liver microsomes from PB-treated rats. Moreover it is not known whether DMB, which is a very lipophilic compound, is itself a substrate for cytochrome P-450. One could therefore infer that the effect of DMB on the carbene spectrum might be secondary to inhibition of \( {\text{CCL}}_4 \) metabolism, thus preventing the formation of the carbene, rather than to a direct scavenging process. This possibility, however, is unlikely from the following information. The observation that lauric acid was a competitive inhibitor of the \( {\text{CCL}}_4 \)-dependent type I binding spectrum with oxidised microsomes (Fig. 4) and had no effect on the carbene spectrum, indirectly suggests that an inhibition of the carbene spectrum by DMB via this mechanism is also unlikely. Furthermore, if the activation of \( {\text{CCL}}_4 \) to reactive metabolites were inhibited by DMB, the loss of haem should have also been prevented, whether \( {\text{CCL}}_3 \) or \( {\text{CCL}}_2 \) was the species responsible, but this was not the case (Table 5).

A further possibility is that the inhibitory effect of DMB on the carbene spectrum may be due to selective inhibition of the formation of \( {\text{CCL}}_2 \) from \( {\text{CCL}}_3 \). One might speculate, for instance, that DMB could modulate the reductive activation of \( {\text{CCL}}_4 \) by limiting the second electron reduction, thus inhibiting \( {\text{CCL}}_2 \) but not \( {\text{CCL}}_3 \) formation. The present results cannot totally exclude this
possibility. Whatever the mechanism of DMB on the \( \text{CCl}_2\text{-P-450} \) spectrum might be, it seems likely from the data available, however, that \( \text{CCl}_2 \) is not significantly involved in the destruction of the cytochrome. This conclusion is also supported by the regeneration of intact cytochrome P-450 from the cytochrome P-450-carbene complex as described by Ahr et al. (1980).

The features of a typical enzymic "suicide" inactivation reaction have been discussed earlier in this thesis (see 3.4.). The present findings clearly indicate a suicide mechanism for the inactivation of cytochrome P-450 by \( \text{CCl}_4 \) under reducing conditions. The calculated partition ratio between metabolic turnover of \( \text{CCl}_4 \) and cytochrome P-450 inactivation (Table 6) indicates that \( \text{CCl}_4 \) is an effective suicide substrate as compared with other compounds (Ortiz de Montellano & Mico, 1981; Loosemore et al., 1981). Values of \( \text{CCl}_4 \) metabolism found in the present study are comparable to those reported in the literature. Ahr et al., (1980) reported a yield of products from \( \text{CCl}_4 \) metabolism by anaerobic NADPH-reduced liver microsomes from PB-treated rats of about 30 nmol/10 min, using \( 1\text{mM} \text{CCl}_4 \). The comparison of this value with the amount of cytochrome P-450 presumably lost under their conditions would have given a value for the partition ratio similar to that calculated in the present study using limiting concentrations of \( \text{CCl}_4 \) (about 27 nmol \( \text{CCl}_4 \) metabolised/nmol cytochrome P-450 destroyed). In their
case the amount of cytochrome P-450 available for suicidal inactivation and not CCl₄, as in our experiment, was limiting. The fact that a similar value was calculated for the partition ratios in two different conditions is consistent with what one would expect to occur for an enzyme inactivation reaction by a suicide substrate (Waley, 1980).

Fig. 32 summarizes the various steps of the reductive metabolism of CCl₄ by microsomal cytochrome P-450 and the related interactions of the products (·CCl₃, :CCl₂ and CO) with the haem prosthetic group. After a one electron reduction of CCl₄ a chlorine anion is released and the free radical ·CCl₃ is formed. This can either be released from the active site of the haemoprotein (1) or bind to the same haem moiety where it was formed, leading to suicidal destruction of the haemoprotein (2), or undergo further reduction to produce :CCl₂ (3). The carbene can then interact with the reduced haem iron leading to reversible inhibition of the cytochrome (4) and/or undergo hydrolysis to CO and HCl. Finally, CO can also bind to reduced cytochrome P-450 haem iron, again resulting in enzyme inhibition (5).

According to this scheme suicidal cytochrome P-450 destruction will occur from the radical generated in the cycle on the right hand side in the Figure, while the enzyme will emerge totally undamaged from the cycle on the left, as noted by Ahr et al. (1980). It should
Fig. 32. Proposed mechanism of interaction of CCl₄ and its metabolites with the haem of reduced cytochrome P-450. Note that two kinds of interaction may occur: the irreversible inactivation of the cytochrome due to destruction of the prosthetic haem probably by the free radical ·CCl₃ or the reversible inhibition of the enzyme due to interaction of its reduced haem with :CCl₂ or CO.
be borne in mind, however, that CCl₄ reactive metabolites produced during the cycle, such as ·CCl₃ and possibly :CCl₂, will bind covalently to other targets than cytochrome P-450 haem, such as protein nucleophiles or lipids. These interactions, however, appear not to influence the loss of cytochrome P-450, at least under anaerobic conditions, since the loss of the cytochrome was found to be equimolar to and, therefore, strictly dependent on, that of microsomal haem.

One final, more general aspect which deserves some consideration is the biological significance of the suicidal process. In other words, what is the relevance for the aerobic in vivo situation of CCl₄-dependent cytochrome P-450 destruction in vitro under anaerobic conditions?

The partial pressure of oxygen in rat liver decreases from the periportal zone (approximately 60-75 mm Hg) to the perivenous zone (approximately 30-40 mm Hg) (Seifter & Englard, 1987; Bell et al., 1980). Local oxygen concentration in the liver in vivo has been reported to vary widely, however, from as low as 1 mm Hg to 60 mm Hg (de Groot & Haas, 1980), with the lower values in the centrilobular region, the location of the higher concentration of cytochrome P-450 and where the hepatic necrosis induced by CCl₄ occurs first. So, reductive activation of polyhalogenated alkanes such as CCl₄ or halothane, does occur in vivo as indicated by the formation of their
volatile metabolites, chloroform (Butler, 1961) and CDE/CTE (Mukai et al., 1977; Sharp et al., 1979), respectively. Suicidal inactivation of specific cytochrome P-450 isoenzymes will probably also occur in vivo under physiological oxygen concentrations leading to a decreased ability of the liver to metabolize some drugs and endogenous substrates.

More difficult is the evaluation of the possible contribution of this process to the complex sequence of events following CCl₄ administration leading to liver necrosis. Different, even contrasting pharmacological and toxicological effects have been demonstrated for, or attributed to, the inactivation of this haemoprotein by suicide substrates in vivo. Some of these are: loss of detoxifying capacity, protection against successive doses of the suicide substrate, induction and inhibition of enzymes involved in haem biosynthesis, immunoallergic reactions, etc. In the case of CCl₄ one can only speculate that a significant role of the suicidal process per se in the overall picture of CCl₄ toxicity cannot be excluded and it is probably largely dependent on the specific conditions under investigation.
7. Conclusions

The results reported in the present thesis show conclusively that haem is both the site and the primary target of the reductive activation of \( \text{CCl}_4 \) by cytochrome P-450, thus demonstrating the working hypothesis (see 1.6).

During reductive metabolism of \( \text{CCl}_4 \) by microsomal cytochrome P-450 several reactive metabolites are formed. Some of these (\( \cdot \text{CCl}_2 \) and CO) interact reversibly with the haem iron and others (probably \( \cdot \text{CCl}_3 \)), attack and inactivate the haemoprotein. The loss of the cytochrome is a typical suicidal process in which the prosthetic haem is the protagonist, whereas the apoprotein only seems to play a secondary role.

Spectrophotometric, chromatographic and radioisotopic studies demonstrated that suicidal, self-catalyzed destruction of haem occurs also in the absence of the apoprotein due to covalent binding of \( \text{CCl}_4 \) reactive metabolites, probably \( \cdot \text{CCl}_3 \), in a 1 to 1 stoichiometric ratio. Essential prerequisites for both enzymatic and non-enzymatic reactions are a free, reduced haem iron and electron donation.
8. References


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THE MECHANISM OF THE SUICIDAL, REDUCTIVE
INACTIVATION OF MICROSMAL 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 (CCl4) 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 CCl4 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 CCl4 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 CCl4 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 CCl4-supplemented microsomal incubations. The results suggest that dichlorocarbene (·CCl2)
is not significantly involved in CCl4-dependent haem destruction.
4. Pretreatment of rats with different microsomal enzyme inducers was responsible for similar but
not identical patterns of CCl4 and CO formation and haem loss during incubation of CCl4 with reduced
microsomes. This indicates a critical role of CCl4 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 CCl4 to ·CCl2 but also the hydrolysis of ·CCl2 to CO.
5. Inactivation of cytochrome P-450 by CCl4 with reduced microsomes from Aroclor-pretreated rats
was saturable and followed pseudo first-order kinetics. This provides further evidence to conclude that
CCl4 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 CCl4 metabolism by liver micro-
somes from Aroclor-pretreated rats has been investigated using limiting concentrations of CCl4. It
was calculated that approximately 26 molecules of CCl4 had to be metabolised to achieve the loss of one
molecule of haem.

During anaerobic metabolism of carbon tetrachloride (CCl4) by NADPH-reduced liver micro-
somes [1, 2] cytochrome P-450 is rapidly inactivated [3, 4] and protohaem, the prosthetic group of cyto-
chrome P-450, is lost [5, 6]. It has been proposed that reactive metabolites such as the trichloromethyl
radical (·CCl3) formed during the reductive microsomal activation of CCl4 [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 CCl4 to
·CCl3, such as those induced by pretreatment of rats with phenobarbitone (PB) or ethanol, are also more susceptible to CCl4-dependent destruction [17, 18]. However, direct evidence as to which reactive
metabolite is responsible for haem and cytochrome P-450 destruction is still missing. CCl4 may undergo
one or two subsequent one electron reductions to form ·CCl2 and dichlorocarbene (·CCl2) respectively.
The latter species has been also identified as a reactive metabolite of CCl4 [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].
In the present study we have investigated the molecular mechanism of the anaerobic CCl₄-dependent destruction of microsomal cytochrome P-450. In particular, the hypothesis that protohaem is the primary, suicidal target of the metabolic activation of CCl₄ 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 CCl₄ during the reductive metabolism of CCl₄ 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-butenone (DMB) was purchased from Aldrich Chemical Co. Ltd. (Gillingham, Dorset, U.K.) and phenobarbitone (PB) from BDH Chemicals. (Poole, Dorset, U.K.). [5-¹⁴C]-amino laevulinic acid ([¹⁴C]-ALA) was purchased from New England Nuclear Research Products (Boston, MA).

[¹⁴C]-Haem (97 mCi/mole) was prepared biosynthetically from [¹⁴C]-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 [¹⁴C]-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 L-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 Na₂HPO₄ buffer, pH 7.4, containing 20% glycerol, flushed with oxygen-free nitrogen (N₂) 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 O₂-free N₂ 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 N₂. The incubation mixture, unless otherwise indicated, contained in 0.1 M Na₂HPO₄ 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), CCl₄ (1 mM) and, where appropriate, DMB (5 mM). The last two reagents were added in methanol. In some experiments MHA containing unlabelled or [¹⁴C]-labelled haem was similarly incubated under anaerobic conditions with CCl₄ 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 emM (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 :CCl₄.** The amounts of CO and :CCl₄ formed from CCl₄ 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 CCl₄ had been added) and a corresponding reference cuvette which contained no CCl₄. 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 :CCl₂–cytochrome P-450 complex (:CCl₂–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 r 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.

**RESULTS**

"**Suicidal**" destruction of cytochrome P-450 by CCl₄ involves parallel destruction of haem prosthetic group

The loss of cytochrome P-450 occurring anaerobically on addition of CCl₄ 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 CCl₄ 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 CCl₄ treatment protohaem undergoes a structural change in its tetrapyrrolic macrocycle. The fluorimetric technique also allowed us to test whether the CCl₄-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 CCl₄-dependent loss of haem was found (Table 1).

**Non-enzymatic "suicidal" activation of CCl₄ by haem**

Since haem itself is readily reduced by sodium dithionite, we investigated the possibility that CCl₄ 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 CCl₄/ 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 [¹⁴C]-haem was incubated anaerobically without any addition (control) or in presence of CCl₄/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 C₁₈ reversed phase column, as described elsewhere [21]. Recovery of injected radioactivity from control and CCl₄/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 CCl₄/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
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 14C-labelled CCl4, approximately 20% of the total radioactivity was eluted in the HPLC fractions containing haem-derived products.

Spectral evidence for CO and :CCl2 formation

When reduced liver microsomes were incubated with CCl4 under anaerobic conditions a difference spectrum appeared (as compared with appropriate controls not treated with CCl4) 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 :CCl2—P-450, shows a peak at 460 nm; the other, due to the complex of CCl4-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 :CCl4—P-450. After incubating PB microsomes with CCl4 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 :CCl2—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 CCl4-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 :CCl4—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.

Effect of a carbene-trapping agent on CCl4-dependent cytochrome P-450 destruction

In order to investigate whether :CCl4 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-
Suicidal cytochrome P-450 inactivation by carbon tetrachloride

Role of CCl₄ metabolism in cytochrome P-450 haem destruction

The effect of several rat pretreatments on CCl₄-dependent reductive destruction of microsomal haem was investigated and related to the production of :CCl₂ 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 :CCl₂, 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 :CCl₂-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 CCl₄-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 CCl₄ (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 CCl₄ 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 CCl₄ 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 CCl₄ had been used. In one of these experiments the total loss of haem obtained with limiting concentrations of CCl₄ (15 nmol/mg protein) was investigated and the effect of additional NADPH and/or CCl₄ was also studied (Fig. 4). Aroclor microsomes were incubated anaerobically at 22° with CCl₄/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 CCl₄-dependent formation of CO and :CCl₂ by NADPH-reduced rat liver microsomes

<table>
<thead>
<tr>
<th>Rat pretreatment</th>
<th>Time (min)</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.035</td>
<td>0.064</td>
<td>0.089</td>
<td>0.107</td>
</tr>
<tr>
<td>:CCl₂</td>
<td></td>
<td>0.392</td>
<td>0.518</td>
<td>0.589</td>
<td>0.696</td>
</tr>
<tr>
<td>(nmol/mg prot)</td>
<td></td>
<td>0.228</td>
<td>0.428</td>
<td>0.518</td>
<td>0.546</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.053</td>
<td>0.071</td>
<td>0.107</td>
<td>0.142</td>
</tr>
<tr>
<td>CO</td>
<td></td>
<td>0.24</td>
<td>0.49</td>
<td>0.83</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.74</td>
<td>1.10</td>
<td>1.52</td>
<td>1.85</td>
</tr>
<tr>
<td>(nmol/mg prot)</td>
<td></td>
<td>0.35</td>
<td>0.82</td>
<td>1.43</td>
<td>1.80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.29</td>
<td>0.55</td>
<td>0.86</td>
<td>1.15</td>
</tr>
<tr>
<td>:CCl₂/CO</td>
<td></td>
<td>0.15</td>
<td>0.13</td>
<td>0.11</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.53</td>
<td>0.47</td>
<td>0.39</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.65</td>
<td>0.52</td>
<td>0.36</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.18</td>
<td>0.13</td>
<td>0.12</td>
<td>0.12</td>
</tr>
</tbody>
</table>

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 Na₂HPO₄ buffer, pH 7.4, Hb (3 μM) and the NADPH generating and oxygen scavenging systems. The reaction was started by injecting CCl₄ 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 :CCl₂ 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 :CCl₂ 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 :CCl₂-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 CCl₄ 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 CCl₄ 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 CCl₄ had to be metabolized on average for every molecule of cytochrome P-450 inactivated.

Table 4. Effect of rat pretreatment on the CCl₄-dependent loss of cytochrome P-450 haem from NADPH-reduced liver microsomes

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Microsomal haem (nmol/mg protein)</th>
<th>Loss of haem (nmol/mg) (% of initial value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>initial</td>
<td>final</td>
</tr>
<tr>
<td>Corn oil</td>
<td>1.48 ± 0.04</td>
<td>1.01 ± 0.07</td>
</tr>
<tr>
<td>PB</td>
<td>2.35 ± 0.10</td>
<td>1.31 ± 0.01</td>
</tr>
<tr>
<td>Aroclor</td>
<td>2.92 ± 0.13</td>
<td>1.37 ± 0.07</td>
</tr>
<tr>
<td>β-NF</td>
<td>1.76 ± 0.02</td>
<td>1.34 ± 0.04</td>
</tr>
</tbody>
</table>

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 CCl₄ (1 mM) in 1.5 ml 0.1 M Na₂HPO₄ 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 CCl₄. 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 CCl₄. Inactivation was biphasic with calculated half-times of 3.2 and 28.9 min. Anaerobic incubations were at 22°C 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 Na₂HPO₄ buffer, pH 7.4 in presence of the oxygen scavenging system described under Materials and Methods. At time 0 CCl₄ (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 b₅, the other haemoprotein present in significant amounts in microsomes, is not vulnerable to CCl₄.

Fig. 4. Haem loss during metabolism of limiting concentrations of CCl₄ by microsomal cytochrome P-450. Experimental conditions and components were as for Fig. 3 but for the initial concentration of CCl₄ in the incubation mixture (15 μM). Control incubation contained NADPH and no CCl₄. As indicated, in 3 parallel incubations additional NADPH (2 μM) and/or CCl₄ (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 CCl₄ 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 CCl₄ metabolism cytochrome P-450 itself, but not NADPH-cytochrome P-450 reductase, the other

### Table 5. Partition ratio between metabolic turnover of CCl₄ and haem inactivation events during suicidal activation of CCl₄ by microsomal cytochrome P-450

<table>
<thead>
<tr>
<th>CCl₄ (nmol/ml)</th>
<th>Haem (nmol/ml)</th>
<th>Partition ratio (CCl₄ metabolized / cyt. P-450 lost)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>initial*</td>
<td>final†</td>
</tr>
<tr>
<td>0</td>
<td>3.05</td>
<td>2.95</td>
</tr>
<tr>
<td>10</td>
<td>3.05</td>
<td>2.56</td>
</tr>
<tr>
<td>15</td>
<td>3.05</td>
<td>2.39</td>
</tr>
</tbody>
</table>

* 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 CCl₄ has been metabolised.
‡ Figures indicate CCl₄-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 Na₂HPO₄ buffer, pH 7.4 in presence of the usual oxygen scavenging system. At time 0 CCl₄ 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 CCl₄ activation [42, 43]. CCl₄-induced lipid peroxidation of the endoplasmic reticulum and covalent binding of CCl₄ metabolites to microsomal lipids and proteins have both been suggested as the mechanisms responsible for CCl₄-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 CCl₄, 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 CCl₄ 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 CCl₄ 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 CCl₄-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 CCl₄-dependent haem destruction is a "suicidal" process requiring the interaction of CCl₄ with a reduced free haem iron [21], probably because the haem iron is involved in enzymatic or chemical transfer of electrons to CCl₄. Thus haem is not only the target but also the site of CCl₄ activation in cytochrome P-450. This view is also supported by the additional finding now reported that haem itself can catalyse its own CCl₄-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 [14C]-haem was incubated reductively with CCl₄, 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 CCl₄ 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 CCl₄ with Fe(II)-tetraphenylporphyrin 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 CCl₄ and metabolites with reduced cytochrome P-450. Note that one electron reduction of CCl₄ produces :CCl₃ 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 :CCl₂ (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). Fe²⁺ = reduced cytochrome P-450, Fe³⁺ = oxidised cytochrome P-450.](image)
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1989

of CCl₄ toxicity has been proposed [45, 46]. The suggestion has also been put forward that the haem moiety of the cytochrome might be alkylated by :CCl₂ [6]. However, if :CCl₂ 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 -CCl₃, the product of one electron reduction of CCl₄.

CCl₄ 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 “discharging” 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 CO and :CCl₂, 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 -CCl₃ from CCl₄ [16, 17]. More direct evidence for a suicidal type of inactivation reaction has now been obtained by showing that the CCl₄-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 CCl₄ by microsomal cytochrome P-450 and related interactions of the products (-CCl₃, :CCl₂ and CO) with the haem of the cytochrome. After a one electron reduction of CCl₄ the free radical -CCl₃ 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 :CCl₂ (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 :CCl₂ 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 CCl₄ 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 CCl₄ is a typical suicide reaction where probably the same molecule of haem responsible for CCl₄ activation is attacked (on average once every 26 catalytic cycles) by a CCl₄ reactive metabolite.

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Enzymatic and/or Non-Enzymatic “Suicidal” Activation of Carbon Tetrachloride by Haem and Cytochrome P-450

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Introduction

During the “suicidal” reductive activation of carbon tetrachloride (CCl₄) by NADPH-reduced rat liver microsomes, cytochrome P-450 is inactivated and haem, the prosthetic group of the cytochrome, is lost (de Groot and Haas, 1981). The loss of haem was found to be equimolar to that of cytochrome P-450 and it is due to irreversible modification of the porphyrin tetrapyrrolic structure (Manno et al., 1986). In a purely chemical system using sodium dithionite as the reducing agent and methaemalbumin (MHA, a water soluble complex of haem with human albumin) as the “suicidal” activator, CCl₄ was also responsible for a dramatic loss of haem and haem-derived protoporphyrin IX (Manno et al., 1987a). Moreover, in preliminary experiments using a mixed system containing both microsomes and exogenous haem in the presence of NADPH, the CCl₄-dependent loss of haem was greater than that found in similar incubations containing microsomes but no MHA (unpublished results), suggesting that exogenous haem was able, in the presence of microsomes, to catalyse its own “suicidal” destruction by promoting the NADPH-dependent activation of CCl₄. The aim of the present study was to investigate the mechanism of the CCl₄-dependent destruction of haem using enzymatic and mixed enzymatic and non-enzymatic systems.

Methods

Male Wistar Albino rats (150–200 g) pretreated with phenobarbitone (PB, 80 mg/kg b.w./day for 3 days, i.p.) were used for microsome preparation as described previously (Manno et al., 1987b). MHA was prepared from proto- or mesohaem as described by Tenhunen et al. (1968). NADPH-cytochrome P-450 reductase
was purified according to Jasukochi and Masters (1976). Cytochrome P-450 was measured by the method of Omura and Sato (1964). Haem was assayed by the pyridine/haemochromogen reaction (Paul et al. 1953) and microsomal protein by the method of Lowry et al. (1951). All incubations were carried out in the presence of an oxygen scavenging system comprising catalase (600 U/ml), glucose oxidase (12.5 U/ml) and D-glucose (60 mM·mM) to ensure anaerobic conditions (below detection by the oxygen electrode) and to prevent lipid peroxidation. Concurrent protohaem and mesohaem determination was performed by a slight modification (Manno and De Matteis, 1986) of the method of Porra (1976), which involves the recording of the absolute sodium dithionite-reduced spectrum between 600 and 500 nm of the incubation mixture containing both haems in a pyridine/NaOH solution. Protohaem and mesohaem concentrations were then obtained by simple algebraic calculations using the extinction coefficients obtained from the absolute spectrum of sodium dithionite-reduced standard solutions.

**Results and Discussion**

When NADPH-reduced liver microsomes from BP-pretreated rats were incubated anaerobically with MHA containing mesohaem — an analog of the physiological protohaem — CCl₄ was responsible for a significant loss of both microsomal protohaem and exogenous mesohaem (Fig. 1). In order to investigate whether under anaerobic conditions NADPH-supplemented rat liver microsomes were able to reduce exogenous haem, oxidised haem (Fe³⁺) and microsomes were incubated together in two identical incubation mixtures in the presence of carbon monoxide (CO) and without CCl₄. The difference spectrum between 500 and 400 nm recorded after the addition of NADPH to the test cuvette gave a peak

![Fig. 1. "Suicidal" loss of cytochrome P-450 protohaem (■) and exogenous mesohaem (○) during their incubation with CCl₄ and NADPH. Anaerobic incubation contained 1.4 mg microsomal protein (1.95 nmol cytochrome P-450/mg), 4.2 nmol mesohaem and 2 µmol NADPH in 2 ml 0.1 M Na₂HPO₄ buffer, pH 7.4, in the presence of the O₂-scavenging system described in Methods. At time 0, 2 µmol CCl₄ was added to test but not to control incubation to start the reaction. Corresponding "control" values for protohaem (□) and exogenous mesohaem (○) are also given.](image-url)
Suicidal Activation of Carbon Tetrachloride

with a maximum at approximately 420 nm which was due to the formation of a reduced haem–CO complex (Fe$^{2+}$–CO). Similar results were obtained when instead of microsomes, purified NADPH-cytochrome P-450 reductase was incubated with MHA (data not shown). In similar incubations but also containing CCl$_4$, approximately 50% of the initial haem underwent “suicidal” CCl$_4$-dependent destruction in 30 min.

The present results indicate at least three different mechanisms for the suicidal activation of CCl$_4$ by haem: enzymatic (1), non-enzymatic (2) and “mixed” (3). In mechanism (1), occurring with microsomes, the electrons required for CCl$_4$ activation are provided to cytochrome P-450 haem by the flavoprotein NADPH-cytochrome P-450 reductase. In (2), which occurs with MHA, CCl$_4$ activation is due to direct reduction of haem in solution by the chemical reductant sodium dithionite. Finally, in mechanism (3), which involves interaction of microsomes with exogenous haem, the electrons provided by NADPH are transferred by microsomal or purified flavoprotein onto exogenous haem, eventually resulting in CCl$_4$ activation and haem loss.

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THE REACTIVE METABOLITES INVOLVED IN THE
ANAEROBIC SUICIDAL DESTRUCTION OF
CYTOCHROME P-450 BY CARBON TETRACHLORIDE

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Introduction

Carbon tetrachloride (CCL4), a well known hepatotoxin, is metabolized anaerobically in vitro to chloroform (CHCl3) by NADPH-reduced liver microsomes. During this reaction cytochrome P-450, the enzyme responsible for CCL4 metabolism, is rapidly inactivated. Protohaem, the prosthetic group of cytochrome P-450, is also lost in the process.

The reactive metabolite directly responsible for cytochrome P-450 inactivation and haem loss is not known. It has been proposed that reactive intermediates such as trichloromethyl radical (:CCL3) or dichloromethylcarbene (:CCL2) formed during the reductive microsomal activation of CCL4 and capable of covalently bind to microsomal lipid and protein, may attack and irreversibly modify the prosthetic group and/or the apoprotein of cytochrome P-450.

In the present study we investigated the molecular mechanism of and the reactive metabolite responsible for the anaerobic CCL4-dependent destruction of microsomal cytochrome P-450. In particular, the hypothesis was tested by which protohaem is both the primary, suicidal target and the site of the metabolic activation of CCL4 by cytochrome P-450. We have also made use of a selective trapping to obtain spectral evidence for the formation of :CCL2 and to study the role played by this reactive intermediate in the process of citochrome P-450 haem destruction.

Materials and methods

Chemicals: Haemoglobin (Hb) from rat blood was prepared by the method of Rossi-Fanelli and Antonini. All other chemicals were obtained from commercial sources and were of analytical grade.

Preparation of microsomes: Male Wistar Albino rats (150-200 g) were pretreated with phenobarbitone (PB) (80 mg/kg/day for three days, ip) dis-
Rats were starved and killed by decapitation 24 h after the last injection. The livers were perfused with saline through the right atrium and the inferior vena cava, removed, minced with scissors and homogenized in four volumes of 0.25 M sucrose in a glass Potter homogenizer. The homogenates were centrifuged at 9,000 g and 4°C for 20 min to obtain the post-mitochondrial supernatant. This was centrifuged at 114,000 g and 4°C for one hour. The microsomal pellet so obtained was resuspended, washed once with 1.15% (w/v) KCl, and ultracentrifuged again as described above. The washed microsomal pellet was finally resuspended in 0.1 M Na₂HPO₄ buffer, pH 7.4, containing 20% glycerol, flushed with oxygen-free nitrogen and stored at -80°C.

Incubations: All incubations were performed anaerobically (below detection by the oxygen electrode) in the presence of an oxygen scavenging system comprising catalase (600 U/ml), glucose oxidase (12.5 U/ml) and D-glucose (60 mM), to prevent lipid peroxidation. The typical incubation mixture, unless otherwise indicated, contained also the following: 1 mg/ml microsomal protein, 1 mM NADPH (or 2 mg/ml sodium dithionite), 1 mM CC1₄ and, when appropriate, 5 mM 2,3-dimethyl-2-butene (DMB).

Assays: Cytochrome P-450 was measured by the method of Omura and Sato. Microsomal haem was assayed by the pyridine/haemochrome method using the reduced absolute spectrum or the reduced minus oxidized spectrum. Microsomal protoporphyrin IX was measured by the oxalic acid method. Microsomal protein was assayed by the method of Lowry et al.

:CCl₃ was measured as described by Ahr et al.

Results

When the loss of cytochrome P-450 and the loss of haem occurring anaerobically on addition of CCl₄ to NADPH-reduced liver microsomes from PB-pretreated rats were compared, a 60% loss of cytochrome P/450 was observed over a period of 5 min and the loss of haem, as measured by the pyridine/haemochrome method, was parallel and equimolar to that of cytochrome P-450 over a period of up to 30 min (Fig. 1).

In order to determine whether the loss of the pyridine-haemochrome reaction was simply due to masking of the haem iron or to a major structural change of the haem moiety, an additional method (i.e. the porphyrin/fluorescence assay) was used to measure haem loss. In this experiment an equimolar CCl₄-dependent loss of microsomal haem and protoporphyrin IX was observed in the same incubations (data not shown), suggesting that protohaem had undergone a major change of the protoporphyrin tetrapyrrolic system. The fluorescence technique allowed also to demonstrate a strong (82%) inhibition
of the haem destruction by 1 mM exogenous carbon monoxide (CO).

![Graph showing loss of cytochrome P-450 and haem](image)

**Figure 1**: CCl₄-dependent loss of cytochrome P-450 and haem during the anaerobic incubation of rat liver microsomes. Incubations were at 37°C in oxygen-free rubber-stoppered glass cuvettes and contained 1 mg/ml liver microsomal protein, a NADPH regenerating system and CCl₄ (1.07 mM) in 2.5 ml 0.1 M Na₂HPO₄ buffer, pH 7.4, containing the oxygen-scavenging system described in Materials and Methods. The reactions were terminated by cooling to 0°C and immediately injecting into the incubation 100 μl of a CO-saturated 50 mg/ml solution of sodium dithionite in 0.1 mM Na₂HPO₄ buffer, pH 7.4 for cytochrome P-450 determination, or by pipetting 0.9 ml of incubation into 2.1 ml pyridine-NaOH mixture for haem assay. When NADPH was omitted the loss of both cytochrome P-450 and haem was negligible over a period of 30 min.

Finally in an attempt to identify the reactive species involved in the destruction of the haemoprotein, the specific carbene-trapping agent DMB was included in a typical incubation mixture. DMB was responsible for a strong (95%) inhibition of the :CCl₄-dependent peak at 460 nm in the difference spectrum of anaerobic dithionite-reduced liver microsomes from PB-pretreated rats (data not shown). This provided additional evidence for the speculated formation of a carbene-cytochrome P-450 ligand complex during the reductive anaerobic metabolism of CCl₄-dependent loss of cytochrome P-450 haem in these incubations (25% and 22% in the absence and the presence of DMB, respectively) nor in NADPH-reduced microsomal incubations (Table 1).
Table 1: Lack of effect of 2,3-dimethyl-2-butene (DMB) on the CCl₄-dependent loss of haem during the anaerobic incubation of reduced rat liver microsomes.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Haem (nmol/mg microsomal protein) (mean ± Sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.63 ± 0.04</td>
</tr>
<tr>
<td>+ NADPH</td>
<td>2.47 ± 0.03*</td>
</tr>
<tr>
<td>+ NADPH and CCl₄</td>
<td>1.62 ± 0.03**</td>
</tr>
<tr>
<td>+ NADPH, CCl₄ and DMB</td>
<td>1.69 ± 0.02**</td>
</tr>
</tbody>
</table>

* p<0.01  
** p<0.001

The anaerobic incubation were at 23°C, for 10 min and contained 1.9 mg microsomal protein (1.95 nmol cytochrome P-450/mg), NADPH (1 mM), CCl₄ (1mM) and DMB (5 mM) in 2 ml 0.1 m Na₂HPO₄ buffer, pH 7.4.

Discussion

The metabolic activation of CCl₄ to reactive intermediates by the cytochrome P-450 system is an essential prerequisite for the hepatotoxicity produced by this toxic agent. It has been known for some time that during both in vivo and in vitro CCl₄ metabolism, cytochrome P-450 itself is inactivated. The parallel and almost equimolar CCl₄-dependent loss of microsomal cytochrome P-450 and haem observed in the present study (Fig. 1) is consistent with the hypothesis that cytochrome P-450 may be damaged due to the direct attack of CCl₄ metabolites onto its prosthetic group haem.²

The possibility was considered here that the observed loss of the pyridine/haemochrome reaction might merely be due to the formation of a reversible ligand complex between the haem moiety and :CCl₂, or other CCl₄ metabolite. In fact, the equimolar loss of microsomal protoporphyrin IX and haem observed in our experiment strongly suggests that the haem moiety has undergone an irreversible change of its tetrapyrrolic ring, due to the attack of CCl₄ reactive metabolites (Fig. 2A). Moreover, the strong inhibition of the protoporphyrin IX loss shown by high concentrations of CO indicates that CCl₄-dependent haem destruction requires the interaction of CCl₄ with a reduced free haem iron. Using a new spectrophotometric method for the concurrent measurement of protohaem and mesohaem in the same sample preparation⁴, we found (unpublished results) that exogenous haem, added to anaerobic microsomal incubations as methaemalbumin (a water-soluble complex of haem with human albumin) may also undergo CCl₄-dependent destruction (Fig. 2A + B). More recently, in an oxygen-free purely chemical system, haem alone was shown to undergo rapid destruction by the reactive intermediates formed during the reductive non-enzymatic activation of CCl₄.
Moreover, in experiments using $^{14}$C-CCL, the amount of radioactivity bound to haem products was calculated to be equimolar to the amount of haem lost. These results as a whole support our hypothesis that haem is both the target and the site of CCL activation by cytochrome P-450 and strongly suggest that cytochrome P-450 destruction is a typical suicidal process.

Figure 2: The "suicidal" reductive destruction of microsomal (A) and exogenous (B) haem by CCL.

The almost complete inhibition induced on the CCL-dependent spectrum by the specific carbene-trapping agent DMB in our incubation provides evidence for the proposed carbene nature of the ligand. However, DMB did not prevent CCL-dependent haem loss, suggesting, by the principle of exclusion, that the free radical, and not the carbene, may be the species responsible for the destruction of cytochrome P-450 haem.

From the present results the following mechanism is proposed for the interaction of CCL reactive with the haem of cytochrome P-450. CCL interacts with the NADPH-cytochrome P-450 reductase-reduced or dithionite-reduced haem iron of cytochrome P-450 and, after an one electron reduction, $^{•}$CCl$_3$ and Cl$^{-}$ are formed. The free radical may either attack the same haem moiety responsible for its formation, leading to suicidal destruction of the haemoprotein or undergo a second one electron reduction to give the carbene. This may also interact with the haem of cytochrome P-450, possibly leading to reversible, non-suicidal inhibition of the haemoprotein.
Conclusions

The evidence described in the present paper indicates that (i) haem is the primary target and the site of the reductive activation of CCl₄ microsomal cytochrome P-450, (ii) the loss of haem is a typical suicidal process which involves irreversible modification of the porphyrin tetrapyrrolic system, and (iii) the free radical, but not the carbene, is likely to be the reactive metabolite of CCl₄ responsible for cytochrome P-450 destruction.

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INTRODUCTION

During the reductive metabolism of carbon tetrachloride (CCl₄) by NADPH-reduced microsomes, cytochrome P-450, the enzyme responsible for CCl₄ activation, is rapidly inactivated and in the process equimolar amounts of protohaem, the prosthetic group of cytochrome P-450, are also lost (de Groot and Haas, 1981). The CCl₄-dependent loss of haem is due to an irreversible change of the tetrapyrrolic ring (Manno et al., 1986). In these experiments the loss of cytochrome P-450 and haem were both significantly prevented by carbon monoxide (CO), suggesting that the destruction of cytochrome P-450 is a suicidal process where haem is both the site of CCl₄ activation and the target of its reactive metabolites.

The evidence presented here indicates that anaerobic haem itself may reductively activate CCl₄ and also undergo suicidal degradation. We have made use of this non-enzymatic model to study the mechanism of haem destruction by CCl₄ and a reverse phase ion pairing h.p.l.c technique has been developed to investigate the products of haem degradation.

MATERIAL AND METHODS

Chemicals and Biochemicals: [¹⁴C]-CCl₄ was purchased from New England Nuclear Research Products (Boston, Mass, USA). Methaemalbumin (MHA), a water soluble complex of haem with human albumin (Sigma), was prepared by the method of Tenhunen et al. (1968) using either unlabelled or ¹⁴C-
Activation of carbon tetrachloride by protohaem

Assays: haem was measured by the pyridine/haemochromogen assay (Paul et al., 1953) and by the oxalic acid method (Morrison, 1965). All incubations were in 0.1M phosphate buffer, pH 7.4, made anaerobic by bubbling with nitrogen and the addition of an oxygen-scavenging system comprising 600 U/ml catalase, 12.5 U/ml glucose oxidase and 60 mM D-glucose.

The H.P.L.C. Technique: A reverse phase ion pairing gradient elution method was developed for separation of haem products from haem. Pigments were extracted from the incubation mixture with two volumes of 80% methanol containing 20mM tetrabutylammonium hydroxide buffered with phosphate (TBA). Extracts were injected and eluted by mixtures of the following solvents: A) 35% methanol in water containing 2.5mM TBA, B) 95% methanol containing 1mM TBA. After one minute elution with 100% A, a 13 min linear gradient to 58.3% A in the A + B mixture was followed by a second linear gradient reaching 100% B in the following 7 min. This concentration was kept constant for the remaining 9 min. Separation was carried out on a column of 30cm x 3.9mm of internal diameter, packed with Bondapak C18 10μm particles (Waters) and fitted with a guard column of 3cm x 3.9mm packed with 30-38μm particles (Whatman).

RESULTS AND DISCUSSION

A rapid loss (84% in 5 min) of haem was observed during the anaerobic incubation at 22°C of 1.6 μM MHA with 1mM CCl4 and 2.9 mM sodium dithionite. The loss of haem in this non-enzymatic system was accompanied by an equimolar loss of its protoporphyrin IX moiety and was almost totally prevented by CO. When protoporphyrin IX itself was incubated with CCl4/dithionite, no loss was observed. These results indicate that, in this non-enzymatic model, like for microsomal incubations, a) haem undergoes drastic modification of the tetrahydroxyl ring, b) a reduced free haem iron is necessary for CCl4 activation.

When [14C]-MHA was incubated anaerobically for 5 min without any addition (control) or with CCl4/dithionite and the radioactivity was extracted in methanol/TBA, the recovery of radioactivity was in both cases quantitative, indicating that volatile products of haem were not formed as a result of CCl4-dependent haem destruction. On injection of these extracts into the h.p.l.c. system, total radioactivity recovered was the same for control and CCl4/dithionite incubations (94 and 93% respectively). Both the absorbance at 400nm and the radioactivity
associated with the haem peak markedly decreased after treatment, but 40-50% of the injected radioactivity was found in several new non-haem fractions eluted immediately before or after haem. These new fractions showed, however, negligible absorbance at 400nm and no fluorescence.

When $^{14}$C-CCl$_4$ was incubated with MHA and sodium dithionite, approximately 20% of the injected radioactivity was found in those h.p.l.c fractions known, from the experiments with $^{14}$C-labelled haem, to contain haem or haem products. When the CCl$_4$-dependent loss of haem and the amount of CCl$_4$ bound to haem products were compared, a one to one stoichiometry was found between haem loss and $^{14}$C-CCl$_4$-derived adduct formation, suggesting that one molecule of CCl$_4$ binds covalently to one molecule of haem.

CONCLUSIONS

1. A non-enzymatic model for the cytochrome P-450-dependent reductive activation of CCl$_4$ has been investigated by using methaemalbumin as a "suicidal" activator.
2. As with cytochrome P-450, the loss of haem caused by CCl$_4$ in this non-enzymatic model was accompanied by equimolar loss of its protoporphyrin IX ring and was prevented by carbon monoxide. Several unidentified products were partially separated from haem by a reverse phase iron pairing h.p.l.c. system and shown to contain radioactivity from prelabelled haem and also from $^{14}$C-labelled CCl$_4$. In addition, the amount of CCl$_4$-derived radioactivity bound to haem products was calculated to be equimolar to the amount of haem lost.
3. We conclude that the destruction of haem caused by CCl$_4$ in both the cytochrome and the non-enzymatic system is a typical suicidal inactivation reaction, where the same molecule of haem responsible for the activation of CCl$_4$ may undergo destruction by a CCl$_4$ reactive intermediate.

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