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CARBON TETRACHLORIDE METABOLISM: THE ROLE OF HEPATIC MICROSOMAL MIXED-FUNCTION OXIDASE COMPONENTS IN CARBON-HALOGEN BOND CLEAVAGE

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By

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ABSTRACT

CARBON TETRACHLORIDE METABOLISM: THE ROLE OF HEPATIC MICROSOMAL MIXED-FUNCTION OXIDASE COMPONENTS IN CARBON-HALOGEN BOND CLEAVAGE

By

Fredrick Oliver O'Neal

Carbon tetrachloride is activated to $\cdot CCl_3$ by the microsomal mixed-function oxidase system. The objectives of this research were (1) to define a role for each mixed-function oxidase component in CCl_4 activation and (2) to determine the mechanisms involved in CCl_4 activation by this system. A model system was developed for determining the role of the flavoprotein, NADPH-cytochrome P_{450} reductase, in CCl_4 activation. The system contained purified reductase, liposomes, and NADPH in the presence or absence of the electron acceptors, ADP-Fe^{+3} and EDTA-Fe^{+3}. The enzyme cycles between its fully and half-reduced states during microsomal electron transfer reactions. Conditions favoring these states could be achieved in the presence and absence, respectively, of the electron acceptors. The CCl_4-dependent stimulation of lipid peroxidation (malondialdehyde formation) or the anaerobic-covalent binding of $^{14}CCl_4$ to liposomes was used to assay CCl_4 activation.

The fully reduced reductase was shown to be incapable of CCl_4 activation. Activation of CCl_4 by the system favoring the half-reduced enzyme was observed. Further investigation of this

activation showed a requirement for lipid hydroperoxides and a pro-oxidant. The reductase converts EDTA-Fe^{+3} to its pro-oxidant form, EDTA-Fe^{+2}, which subsequently degrades lipid hydroperoxides to lipid radicals. These radicals activate CCl_4 by an atom transfer reaction. Cytochrome $P_{450}(Fe^{+3})$ as well as hemin and other oxidized hemoproteins will also serve as pro-oxidants in this system. It was concluded that the reductase does not activate CCl_4. However, it functions indirectly via its ability to reduce cytochrome P_{450} and to form lipid hydroperoxides from polyunsaturated fatty acids when ADP-Fe^{+3} and O_2 are present. The degradation of these hydroperoxides by pro-oxidants results in CCl_4 activation.

The role of cytochrome P_{450} in CCl_4 activation was investigated in two systems. The first was liver microsomes from rats pretreated with $CoCl_2$ to alter cytochrome P_{450} levels. The second was a reconstituted system containing liposomes, cytochrome P_{450}, and the reductase. In microsomes, both the rate and extent of CCl_4-stimulated lipid peroxidation were related to the cytochrome P_{450} content. In the reconstituted system, an NADPH-dependent binding of $^{14}CCl_4$ was inhibited by an antibody to cytochrome P_{450} and by CO. Binding also resulted when dithionite was used as a source of reducing equivalents for cytochrome P_{450}. Dithionite-reduced hemoglobin and hemin also activated CCl_4. It was concluded that cytochrome P_{450} is the site of CCl_4 activation within the mixed-function oxidase system. Also, the interaction of CCl_4 with the reduced heme iron of cytochrome P_{450} is required for CCl_4 activation to $\cdot CCl_3$.

Liver microsomes from untreated rats or those pretreated with PB and 3-MC were utilized to determine the role of different cytochrome P_{450} hemoproteins in CCl_4 activation. The initial rates of CCl_4-stimulated lipid peroxidation and $^{14}CCl_4$ binding were expressed on the bases of microsomal cytochrome P_{450} content, turnover numbers. These values were consistently higher for untreated microsomes; however, the relative magnitudes of these numbers for PB- and 3MC-microsomes depended on the method used to assay CCl_4 activation. These differences are believed due to the relative amounts of $\cdot CCl_3$ and $:CCl_3$ produced by each microsomal cytochrome P_{450} population. The former initiates lipid peroxidation as well as binds to lipid whereas the latter is only capable of binding. The turnover numbers for $^{14}CCl_4$ binding were more dependent on the reductase to cytochrome P_{450} ratios than on cytochrome P_{450}'s substrate specificity. This was confirmed by the fact that reconstituted systems containing the same amount of reductase and either cytochromes P_{450} or P_{448} gave similar rates of $^{14}CCl_4$ binding.

In conclusion the following factors may account for observed differences in CCl_4 activation and susceptibility of tissues to toxicity: the microsomal cytochrome P_{450} content and its rate of reduction; the relative amounts of $\cdot CCl_3$ or other toxic intermediates formed <u>in vivo</u>; and cellular protection mechanisms.

To My Parents

ACKNOWLEDGMENTS

I would like to express my appreciation to Dr. Steven D. Aust, my academic advisor, for the many contributions made during my graduate work. His welcome of a rather weathered sailor back into his laboratory and his guidance and concern during this phase of my scientific career are especially appreciated. It is with gratitude that I acknowledge the assistance of my Uncle Sam. His providing me with the opportunity to acquire those sailing skills and financial assistance has made weathering the fiscal storm of graduate school bearable. I also wish to express my thanks to other members of my guidance committee, Drs. Bruce A. Averill, David G. McConnell, William L. Smith, and John E. Wilson for their helpful discussions. I would also like to acknowledge the following members of the Aust Laboratory for their assistance and many helpful discussions: Drs. John Buege, Robert Moore, and Ann Welton; Mr. Bruce Svingen; and Mrs. Poonsin Olson. Finally, I wish to acknowledge other members of the laboratory and of the Biochemistry Department for their moral support and friendship.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
BP	3,4-Benzpyrene
BHT	Butylated hydroxytoluene
CHP	Cumene hydroperoxide
Ci	Curie
cpm	Counts per minute
DEAE	Diethylaminoethyl
di-1,2-GPC	Dilauroylglyceryl-3-phosphoryl choline
DDT	1,1,1-Trichloro-2,2,-bis-(p-chlorophenyl)ethane
EDTA	Ethylenediaminetetraacetate
G6Pase	Glucose-6-phosphatase
i.p.	Intraperitoneal
IgG	Immuno gamma globulins
LOOH	Lipid hydroperoxides
MDA	Malondialdehyde
MeOH	Methanol
3-MC	3-Methycholanthrene
NADH	β-Nicotinamide adenine dinucleotide, reduced form
NADPH	β-Nicotinamide adenine dinucleotide phosphate, reduced form
PB	Phenobarbital

pCMB	p-Chloro mercuribenzoate
PCN	Pregnenolone-16α-carbonitrile
PEG	Polyethylene glycol
s.c.	Subcutaneous
S.D.	Standard deviation
SDS	Sodium dodecyl sulfate
SKF-525A	2-Diethylaminoethyl-2,2-diphenylvalerate
Tris	Tris(hydroxy methyl) amino methane

INTRODUCTION

Background of the Study

Halogenated alkanes are quite prevalent and are being used for purposes ranging from medicinal to industrial. Millions of tons of these compounds are produced yearly and we are by various mechanisms becoming increasingly exposed. It is of concern that many of these compounds, once believed to be innocuous, have been shown to be either toxic or can undergo metabolic activation into compounds that are. For example, several of the halogenated methanes which have been used as anesthetics, fire extinguishants, industrial solvents, and as aerosol propellants were once believed to be quite stable and not readily metabolized. That they are metabolized has been amply demonstrated and a correlation made between their ability to form reactive intermediates and toxicity. Also, two of these halomethanes, CCl_4 and $CHCl_3$, have been shown to be carcinogenic in some laboratory animals.

The hepatic microsomal mixed-function oxidase system is responsible for the metabolism of a variety of foreign compounds as well as endogenous substrates. It also has a major role in the dehalogenation and subsequent activation of haloalkanes. The mechanisms involved in the dehalogenation of these compounds to active intermediates and their subsequent interaction with cellular compounds leading to cell death are poorly understood. However,

it has been demonstrated that the metabolism of some of these compounds initiates lipid peroxidation, a deleterious free radical mediated process, in microsomal membranes. Because of my general interest in toxicology and our laboratory's involvement with enzyme-catalyzed lipid peroxidation, I became interested in investigating the reactions involved in activating these compounds by the microsomal mixed-function oxidase system.

The general approach taken in these investigations was to characterize the metabolism of a model haloalkane in reconstituted systems containing purified mixed-function oxidase components. Carbon tetrachloride was suitable for these investigations because many of the conditions affecting its metabolism and toxicity had been determined and several metabolites identified. The metabolic activation of this compound initiates lipid peroxidation both _in vitro_ and _in vivo_; there is also evidence suggesting the trichloromethyl radical ($\cdot CCl_3$) is the metabolite responsible for this initiation. Unlike most of the reactions catalyzed by the microsomal mixed-function oxidase system, the metabolism of CCl_4 to $\cdot CCl_3$ involves a reductive mechanism which can take place in the absence of oxygen. Only two reactions catalyzed by this system involving reductive mechanisms have been characterized, the reduction of compounds containing azo and nitro groups to their corresponding amines. Reducing equivalents can be transferred to the azo and nitro moieties of the compounds from both the flavoprotein, NADPH-cytochrome P_{450} reductase and the reduced hemoprotein,

cytochrome P_{450}, components of the hepatic microsomal mixed-function oxidase system. Similarly, evidence exists which suggests that either one or both of the mixed-function oxidase components are required for CCl_4 activation, however; direct proof is lacking.

Chapters 2 and 3 of this thesis include the investigations of the functional roles that NADPH-cytochrome P_{450} reductase and cytochrome P_{450}, respectively, have in CCl_4 activation. These investigations have been made possible by improvements in purification techniques for these components and the ability to reconstitute mixed-function oxidase activity. Model systems containing liposomes and one or both of the purified components have been developed to evaluate their participation in CCl_4 activation free of interference from other microsomal constituents.

The cytochrome P_{450} component of the microsomal mixed-function oxidase system of the liver represents a family of hemoproteins with different but sometimes overlapping substrate specificities. The preferential induction of one or more of these hemoproteins by pretreatment with drugs has been shown to alter CCl_4 toxicity. For example, PB pretreatment increases whereas 3-MC decreases toxicity when compared to the controls. One possible explanation for these observed differences is that these hemoproteins differ in their ability to activate CCl_4. Chapter 4 investigates this possibility in microsomes as well as model systems containing catalytically different cytochrome P_{450} hemoproteins.

Although there are several chemical reactions which form $\cdot CCl_3$, it has not been determined how this may occur enzymatically. Chapters II and III include the investigations to determine the reactions in which the reductase and cytochrome P_{450} components participate to activate CCl_4. Liposomal systems containing purified components were used here also.

Organization of Dissertation

This dissertation is divided into five chapters; the first and last contain the Literature Review and Summary, respectively. The investigations of CCl_4 activation by the hepatic microsomal mixed-function oxidase system have been divided into three areas. Each area of investigation is presented separately in Chapters II through IV with a format similar to that used in many scientific papers. These chapters are subdivided into the following sections: Abstract, Introduction, Materials and Methods, and Discussion. Chapter references as well as those for the Literature Review section have all been combined at the end of the dissertation. The Literature Review section presents a broad overview of the current knowledge concerning the function of the microsomal mixed-function system and its role in CCl_4 metabolism and toxicity. A broad overview of lipid peroxidation is also presented since it is believed to have a key role in CCl_4 toxicity. The introduction to each chapter is designed to provide a more specific background for that aspect of the research. Chapter V summarizes the data presented in Chapters II through IV.

CHAPTER I

LITERATURE REVIEW

Microsomal Mixed-Function Oxidase System

Mixed-function oxidases are generally defined as enzymes which catalyze the consumption of one molecule of oxygen per molecule of substrate with one oxygen atom being incorporated into the product and the other reduced to water (Mason, 1957). The endoplasmic reticulum (microsomes) of rat hepatocytes has a membrane-associated mixed-function oxidase system which is involved in the metabolism of a broad spectrum of lipophilic substrates (Conney, 1967; Kuntzman, 1969; Mannering, 1971). Among these are the normal body constituents, steroids and fatty acids, and foreign compounds (xenobiotics) which include drugs, pesticides, carcinogens, industrial chemicals and food additives. In rat liver microsomes, mixed-function oxidase activity is associated with the NADPH-dependent electron transport chain. This chain contains two components: cytochrome P_{450}, a hemoprotein so designated because of the characteristic absorption maximum at 450 mm for its reduced-CO difference spectrum (Omura and Sato, 1964), and NADPH-cytochrome P_{450} reductase, a flavoprotein. The reductase transfers reducing equivalents from NADPH to cytochrome P_{450} which serves as the terminal oxidase for the system. In addition to oxygen, two

general classifications of substrates will bind to cytochrome P_{450}, Types I and II (Mannering, 1971). When bound to the oxidized (Fe^{+3}) form of the hemoprotein each class of substrates will give its characteristic difference spectra. The binding sites for these substrate types are different with the Type II binding site being either at or closely associated with the heme moiety whereas the Type I binding site is not. Cytochrome P_{450} serves as the site for the formation of "active oxygen" which is subsequently incorporated into substrates of both classifications. A brief summary of the sequential events involved in cytochrome P_{450}-mediated oxidations include: formation of a cytochrome $P_{450}(Fe^{+3})$-substrate complex; a one electron reduction of the complex by NADPH-cytochrome P_{450} reductase; oxygen binding to the reduced heme iron of cytochrome P_{450} forming a ternary complex; transfer of a second electron to form a short-lived "active-oxygen" complex; and the dissociation of the latter into the products, hydroxylated substrates and water, and cytochrome $P_{450}(Fe^{+3})$ (Estabrook, 1973).

Cytochrome P_{450}-containing mixed-function oxidases are of widespread occurrence in nature. They have been found in bacteria, yeast, plants, insects, fish, and mammals (Capdevila and Agosin, 1977; Katagiri et al., 1968; Mannering, 1971; Yoshida et al., 1977). In mammals mixed-function oxidase activity is greatest in liver microsomes but has been found also in microsomes from the kidney, lung, brain, placenta, intestine, and testis, and in adrenal cortex mitochrondria (Conney, 1967; Sasame et al., 1977; Villarruel et al., 1977).

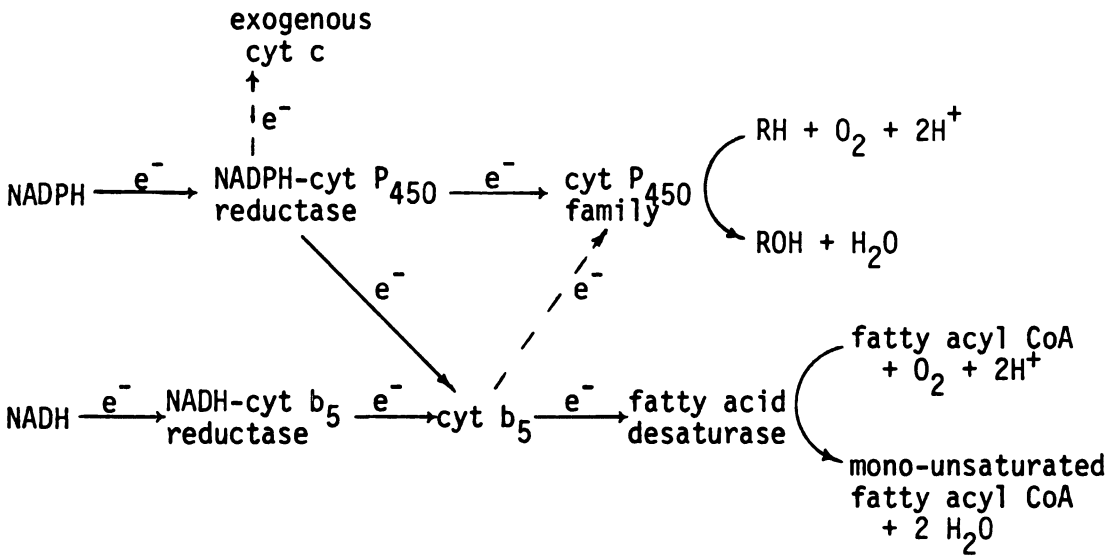
The mixed-function oxidase system of the liver functions primarily in the conversion of its lipophilic substrates, xenobiotics, to more water-soluble forms which facilitates their excretion into the urine, bile, and expired air (Cafruny, 1971; Munsen and Eger, 1971; Plaa, 1971). A wide variety of oxidative reactions are involved in the metabolism of these substrates. Included among these are the hydroxylation of aliphatic and aromatic compounds, epoxidations, O- and N-dealkylations, N-oxidations, sulphoxidations and desulphurations (Mannering, 1971; Parke, 1975). Although the reaction products are varied, one unifying concept has been that these are all different types of hydroxylation reactions. Some of these reactions form unstable intermediates that quickly rearrange to the more stable products (Brodie et al., 1958). Reductive reactions, for example, the azo- and nitro-reductases, are also catalyzed by this system (Gillette, 1971a). The products of both the oxidative and reductive reactions may be either excreted directly or further metabolized. Microsomal and cytosolic enzymes can catalyze the conjugation of many of these products with more water-soluble compounds such as glucuronic acid, glutathione, and sulfate (Mandel, 1971; Shuster, 1964).

In general the reactions catalyzed by the microsomal mixed-function oxidase system function to detoxify xenobiotics but, unfortunately, they also have the capacity to increase the toxicity of relatively innocuous substances. Many cancerous agents and cellular oxidants are formed <u>in vivo</u> from the relatively harmless pro-carcinogens and pro-oxidants, respectively

(Jerina and Daly, 1974; Plaa and Witschi, 1976). Another characteristic of the hepatic as well as other mixed-function oxidase systems is that they may be induced up to several fold by various compounds (Conney, 1967; Parke, 1975). The compounds which induce these enzymes are classified as either general or specific inducers depending on their abilities to induce a broad or limited range of activities (Conney, 1967; Gillette, 1971b). Phenobarbital (PB) is the classical prototype of the general inducers causing a proliferation of the cell's endoplasmic reticulum as well as an increase in its cytochrome P_{450} and cytochrome P_{450} reductase components. The polycyclic hydrocarbon, 3-MC, is the prototype for the specific inducers which in general only increase the hemoprotein component whose reduced-CO difference spectrum has its absorbance maximum shifted to 448 nm. The induction of mixed-function oxidase activity has several important consequences which include an increased tolerance to therapeutic drugs, altered metabolism of steroid hormones, and increased activation of the potentially toxic substances, pro-carcinogens and pro-oxidants (Hall, 1976; Jerina and Daly, 1974; Plaa and Witschi, 1976).

There are two electron transport chains associated with rat liver microsomes, the NADPH-dependent chain previously described and an NADH-dependent chain which functions in the desaturation of fatty acids (Oshino, 1966, 1970; Strittmatter, 1974). The components of this chain include the flavoprotein, NADH-cytochrome b_5 reductase, cytochrome b_5, and the desaturase enzyme. Although these

two electron transport chains have dissimilar functions, they are believed to interact as shown schematically below:



The NADPH-dependent chain contains NADPH-cytochrome P_{450} reductase and a family of cytochrome P_{450} hemoproteins which confer broad substrate specificity on the mixed-function oxidase system. The reductase can transfer electrons to exogenous acceptors such as cytochrome c and various forms of chelated iron and to the endogenous acceptors, cytochromes P_{450} and b_5 (Archakov et al., 1975; Noguchi and Nakano, 1974; Pederson et al., 1973; Yasukochi and Masters, 1976). Although cytochrome b_5 is reduced by NADPH-cytochrome P_{450} reductase, the rate is much slower than that involving NADH-cytochrome b_5 reductase (Archakov et al., 1975). Microsomal mixed-function oxidase reactions involve a two electron reduction of molecular oxygen and it has been hypothesized by Hildebrandt and Estabrook (1971) that cytochrome b_5 serves as the source of the second electron.

Several lines of evidence suggest that reducing equivalents are transferred from the reduced cytochrome b_5 to cytochrome P_{450}. These include the following: the NADH/NADPH synergism in the microsomal metabolism of several substrates (Cohen and Estabrook, 1971; Mannering, 1974); the substrate-dependent increase in cytochrome b_5 oxidation (Fujita and Peisac, 1977; Mannering, 1974); and the metabolism of 3,4-benzpyrene in a reconstituted system containing cytochrome b_5, NADH-cytochrome b_5 reductase, lipid and cytochrome P_{448} (Lu et al., 1974a). Although some interaction between the two microsomal electron transport chains has been demonstrated in vitro, some uncertainty still exists concerning the function of this interaction in vivo (Schenkman and Jansson, 1974). The metabolism of several drugs by a reconstituted system containing only cytochrome P_{450} and cytochrome P_{450} reductase suggested that cytochrome b_5 was not an obligatory component of the microsomal mixed-function oxidase system (Guengerich, 1977).

Purification of Mixed-Function Oxidase Components

Mixed-function oxidase components are tightly associated with microsomal membranes. Until recently, this property has made them difficult to purify and characterize. Earlier attempts at purification led to either a deactivation or incomplete resolution of these components from other membrane proteins and lipids. However, newly developed techniques have led to the purification of the cytochrome P_{450} and NADPH-cytochrome P_{450} reductase components in

forms that retain their catalytic activity. The use of hydrophobic (Shaltiel, 1975) and affinity (Cuatrecasas, 1970) chromatography in the presence of ionic and non-ionic detergent mixtures has made possible the purification of both components in good yields (Guengerich, 1977; Imai, 1976; Yasukochi and Masters, 1976). SDS-polyacrylamide gel electrophoresis utilizing both heme and protein staining techniques has been a very useful analytical tool in determining the molecular weights and homogeneity of purified mixed-function oxidase components and has aided in the identification of the cytochrome P_{450} hemoproteins (Fairbanks, 1971; Moore et al., 1978).

NADPH-cytochrome P_{450} reductase had been solubilized and purified earlier by proteolysis (Williams and Kamin, 1962; Pederson et al., 1973) and more recently by affinity chromatography in the presence of non-ionic detergents (Yasukochi and Masters, 1976). Both procedures resulted in enzymes with similar flavin contents and each was able to reduce the exogenous substrates, cytochrome c and $K_3Fe(CN)_6$. The major differences between the two preparations were that the protease-solubilized reductase was approximately 8,000 daltons smaller and had lost its ability to reduce cytochrome P_{450} (Lu et al., 1969; Welton et al., 1973). The proteolysis of microsomal membranes separates the catalytically active hydrophilic region of cytochrome P_{450} reductase, an amphipathic molecule, from the non-catalytic hydrophobic region. The latter functions as an anchor attaching cytochrome P_{450} reductase to the hydrophobic

microsomal membrane matrix (Peterson et al., 1976) and probably functions similarly in reconstituted systems, facilitating its reduction of cytochrome P_{450} in the liposomal membranes. The detergent-solubilized reductase is capable of reducing not only different cytochrome P_{450} hemoproteins from the same animal but those of other species as well (Coon et al., 1977; Lu and Levin, 1974).

It was generally believed that NADPH-cytochrome P_{450} reductase was a single enzyme (Welton, 1974); however, Coon et al. (1977) have recently obtained evidence for two forms in rabbit and rat liver microsomes. Two detergent-solubilized NADPH-cytochrome P_{450} reductases have been isolated from the liver microsomes of both PB pretreated species. Although the purification methods for each form were different, they resulted in homogeneous preparations with similar flavin contents but different specific activities when cytochrome c was used as the electron acceptor and different electrophoretic mobilities during SDS-polyacrylamide gel electrophoresis. Microsomal proteins with electrophoretic mobilities corresponding to molecular weights of 78,000 and 76,000 daltons in the rat and 74,000 and 68,000 daltons in the rabbit were found to be identical to those of the purified reductases. Both NADPH-cytochrome P_{450} reductases were present in untreated and PB microsomes in equal amounts suggesting that neither form was preferentially induced. Both forms could also reduce several cytochrome P_{450} hemoproteins. Neither gel pattern was affected when the microsomes or enzyme preparation contained the general protease inhibitor, phenylmethane

sulfonyl fluoride. Trypsin treatments of the purified proteins convert both rat enzymes to a 69,000 dalton species, lower than either detergent-isolated form. Both findings suggest proteolytic artifacts during isolation procedures are not responsible for the different reductases. The existence of more than one cytochrome P_{450} reductase has interesting implications. In vivo, where the reductase component may be limiting, preferential reduction of cytochrome P_{450} hemoproteins by specific reductases would greatly affect the overall rate of metabolism for specific substrates. This matter is the subject of further investigation by that laboratory.

Cytochrome P_{450} has been the mixed-function oxidase component most difficult to purify. Earlier attempts at purification either resulted in incomplete resolution from membrane proteins or its conversion to the inactive, cytochrome P_{420}, form (Lu and Levin, 1974). Techniques for purifying this hemoprotein in a catalytically active form have now been developed in several laboratories (Coon et al., 1977; Guengerich, 1977; Lu and Levin, 1974). Generally, these procedures involve solubilization with ionic detergents, initial fractionation by either ammonium sulfate, polyethylene glycol, or hydrophobic chromatography, and further purifications involving ion exchange chromatography in the presence of non-ionic detergents.

It is generally accepted that multiple cytochrome P_{450} hemoproteins exist and that this accounts for the rather broad

substrate specificity of the microsomal mixed-function oxidase system. Using various drug pretreatments and the above purification procedures, Guengerich (1977) has been able to demonstrate at least eight cytochrome P_{450} hemoproteins in the rat liver microsomes and at least six in rabbit liver microsomes. Neither should be taken as a final number in that more are likely to be found as techniques improve. Of the cytochrome P_{450} hemoproteins purified to date, differences have been found in some but not all of the following: molecular weight; amino acid content; substrate specificity (most were found to have overlapping but different substrate specificities); pH optima for reconstitution of mixed-function oxidase activity; antigenic determinants; and reduced-CO difference spectra (Coon et al., 1977; Guengerich, 1977; Lu et al., 1976).

Reconstitution of Mixed-Function Oxidase Activity

Our understanding of the metabolism of drugs by the NADPH-dependent mixed-function oxidase system has been greatly increased by the successful reconstitution of its activity from purified components. Lu et al. (1969) found a requirement for a heat-stable lipid fraction in addition to partially purified cytochrome P_{450} and cytochrome P_{450} reductase for mixed-function oxidase activity. It was later determined that this fraction could be replaced by synthetically prepared phosphatidyl cholines or by detergents (Lu et al., 1974b; Strobel et al., 1970). The lipid fraction facilitates the reduction of cytochrome P_{450}, probably by mimicking the

hydrophobic membrane matrix of microsomes allowing for proper positioning and/or protein conformations favorable for electron transfer (Lu et al., 1974b; Lu and Levin, 1974). The order of mixing the mixed-function oxidase components and lipid is important. Maximum rates of drug metabolism are achieved only when the lipid, cytochrome P_{450} reductase, and cytochrome P_{450} fractions are thoroughly mixed prior to the addition of the buffer and other cofactors (Lu and Levin, 1974).

Several investigators have now been able to reconstitute mixed-function oxidase activity from components purified from a variety of sources (Coon et al., 1977; Kamataki et al., 1976; Lu and Levin, 1974). The substrate specificities and reaction products are similar to those of the microsomes from which the cytochrome P_{450} components were isolated. It was observed that the ratio of the protein components, reductase to cytochrome P_{450}, required for maximum activity in these reconstituted systems was generally higher than that found in microsomes (Kamataki et al., 1976; Lu et al., 1972). Cytochrome P_{450} reductase is the limiting component in microsomes generally present at a ratio of one molecule of reductase to twenty molecules of cytochrome P_{450} (Peterson et al., 1976). Most of the reconstituted systems contain cytochrome P_{450} as the limiting component. This is reflected in the fact that turnover numbers, mole of product formed per minute per mole of cytochrome P_{450}, are higher for a number of substrates in the

reconstituted system than in microsomes (Lu and Levin, 1974; van der Hoeven and Coon, 1974).

The reconstitution of microsomal mixed-function oxidase activity has provided a very useful tool for investigating the metabolism of a variety of compounds. Information regarding the specificity, product formation, and mechanisms involved in the metabolism of substrates in mixed-function oxidase systems containing various cytochrome P_{450} hemoproteins can be obtained. A means is also provided for the evaluation of requirements for other microsomal constituents in mixed-function oxidase reactions. Mixed-function oxidase as well as other reconstituted membrane systems will be used in several investigations reported in this thesis.

Carbon Tetrachloride Metabolism and Toxicity

Carbon tetrachloride is one of several halogenated alkanes which has had many useful applications within the last century. Included among these applications have been its use as an industrial and dry-cleaning solvent, a fire-extinguishing agent, an anesthetic, and as an antihelminthic agent for the treatment of hookworms (von Oettingen, 1955). The toxicity of this compound has been well established and the symptoms of its poisoning include the following: severe liver and kidney dysfunction; central nervous system depression; increased clotting time; decreases in lymphocytes, leucocytes, and blood platelets (Bini, 1976; Back, 1977; von Oettingen, 1955). Although the

effects of CCl_4 poisoning are apparent to some degree in other tissues, the most severe damage occurs in the liver. Because the CCl_4-induced liver damage is similar to that caused by other hepatotoxins and by certain forms of liver diseases, CCl_4 is often used as a model compound in investigations of mechanisms for general liver dysfunction (Goldblatt, 1972; Rees, 1976).

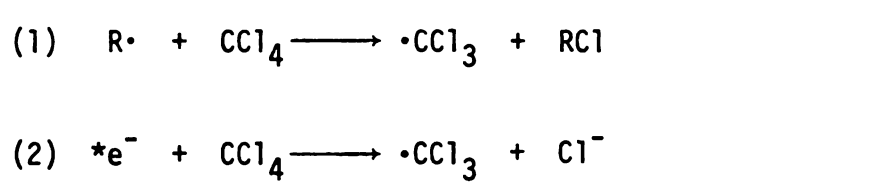
Several theories had previously been developed to account for CCl_4 toxicity; however, it is now generally accepted that its metabolism to a reactive intermediate by the microsomal mixed-function oxidase system is required (Recknagel and Glende, 1973; Recknagel, 1967). Some of the observations leading to this hypothesis are briefly mentioned below. Exposure of laboratory animals to $^{14}CCl_4$ leads to the expiration of small amounts of $^{14}CO_2$ and to the covalent binding of the label to cellular lipids and proteins (Benedetti et al., 1977; Gillette et al., 1974; Gordis, 1969; Villarruel and Castro, 1975). Cytotoxicity occurred only in those tissues with high mixed-function oxidase activity, e.g., the liver and kidney, even though significant amounts of CCl_4 were present in other tissues as well (Villarruel, 1971). Animals such as chickens which are resistant to CCl_4 toxicity generally have low hepatic microsomal mixed-function oxidase activity (Diaz Gomez et al., 1975; Recknagel and Glende, 1973). The evidence which more directly implicates the microsomal mixed-function oxidase system includes the following: initial damage is localized within the microsomes resulting in a loss of cytochrome P_{450}, G6Pase,

membrane lipids, and alteration of other microsomal functions; general inducers of mixed-function oxidase activity increase toxicity whereas its inhibitors decrease toxicity; and that CCl_4 forms a Type I substrate binding spectrum when added to microsomes, suggesting the involvement of cytochrome P_{450} in its metabolism (Recknagel and Glende, 1973).

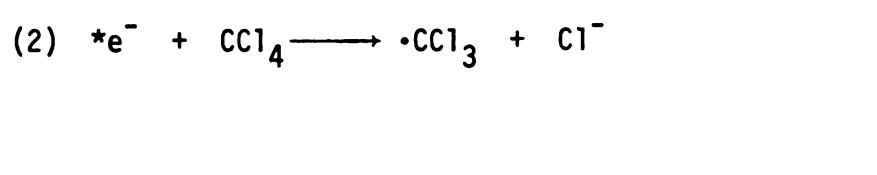
Characterization of the products of CCl_4 metabolism has been undertaken by several investigators to determine the identity of its toxic intermediate. The discovery that free radical trappers and antioxidants protect animals from the toxic effects of CCl_4 led to the suggestion that this intermediate was a free radical (Recknagel, 1967; Reynolds and Moslen, 1974). Recknagel and Ghoshal (1966) demonstrated that CCl_4 increased NADPH-dependent microsomal lipid peroxidation, a free radical mediated process *in vitro*. They also demonstrated a CCl_4-dependent increase in the conjugated diene byproduct of lipid peroxidation in microsomes from CCl_4-treated rats. This provided the first indication that CCl_4-dependent lipid peroxidation occurred *in vivo*. Confirmation of this finding came later when the ethane and pentane byproducts of lipid peroxidation were found in the expired breath of CCl_4-treated rats (Riely et al., 1974). Pretreatment of these animals with antioxidants decreased alkane evolution and a correlation was found between the CCl_4-dependent expiration of alkanes and hepatic mixed-function oxidase activity (Dillard et al., 1976; Hafeman and Hoekstra, 1977; Lindstrom and Anders, 1978; Riely et al., 1974).

The detection of significant quantities of $CHCl_3$ and CCl_3-CCl_3 in animals exposed to CCl_4 led to the hypothesis that the trichloromethyl radical ($\cdot CCl_3$) was the toxic intermediate (Bini et al., 1975; Fowler, 1969). Chloroform would be the expected product of the abstraction of a hydrogen atom from cellular components by $\cdot CCl_3$. Hexachloroethane would be the product resulting from the dimerization of two $\cdot CCl_3$. Both reactions are common to many free radical mediated processes (Pryor, 1976). More recently, Villarruel and Castro (1975) chemically generated $\cdot CCl_3$ in the presence of polyunsaturated fatty acid methyl esters and demonstrated a similarity between the labeled products and those isolated from rats previously injected with $^{14}CCl_4$. This binding could occur by two reactions, one involves a termination reaction with a lipid radical and the other, an addition to the olefin bonds resulting in a trichloromethylated lipid radical (Benedetti et al., 1977).

It is now generally accepted that $\cdot CCl_3$ formation does occur during CCl_4 metabolism; however, the reactions involved <u>in vivo</u> have not been characterized. Chemical reactions which catalyze $\cdot CCl_3$ formation are known; those which may be biologically important are as follows (Gregory, 1966; Pryor, 1966):



(1) $R\cdot + CCl_4 \longrightarrow \cdot CCl_3 + RCl$



(2) $*e^- + CCl_4 \longrightarrow \cdot CCl_3 + Cl^-$

Since free radicals are normal intermediates of many biological reactions, activation may occur by the "atom-transfer" reaction in equation (1). It is of interest that several flavoproteins form the flavin semiquinone radical intermediate in many of their reactions (Dixon, 1971b; Iyanagi et al., 1974; Slater, 1972). The possible activation of CCl_4 by the flavoprotein NADPH-cytochrome P_{450} reductase by this reaction will be one of the questions addressed in this thesis. Reaction (2) is that proposed by Reynolds (1966) who coined the term "electron capture" to describe its mechanism. This reaction has been demonstrated to take place in the gas phase with a source of "high-energy" electrons available. Reynolds has suggested that this reaction could occur in biological systems and that the source of electrons would be those "loosely bound" to cellular electron transfer components. Support for this hypothesis stems from the observations that the reduced forms of some transition metals, heme compounds, and hemoproteins interact with various halogenated compounds yielding free radical intermediates (Asscher and Vofsi, 1963; Stotter et al., 1977; Wade and Castro, 1973; Wade et al., 1969). The implications of these findings with regard to CCl_4 activation by the microsomal mixed-function oxidase system will be discussed in more detail later.

Reactions (1) and (2) are similar to the chemical reactions which involve classical S_N2 mechanisms (Morrison and Boyd, 1971). Pryor (1976) has used the designation S_H2, for substitution, homolytic-bimolecular, to distinguish it from that

involving the nucleophilic reactant. A bimolecular intermediate complex may be formed between CCl_4 and either a free radical or cellular electron transfer component. Dissociation of this intermediate would involve cleavage of a carbon-halogen bond and subsequent $\cdot CCl_3$ formation. The extent to which this reaction occurs would to a large part be dependent on the dissociation energy of the bond being broken. That reactions (1) and (2) may be biologically significant is further supported by the relationships between the hepatotoxicity of compounds in the chloromethane series and their bond dissociation energies (Recknagel and Glende, 1973; Reynolds and Yee, 1967). In order of decreasing C-Cl bond dissociation energy, the toxicity of halomethanes increases as follows:

$$CH_3Cl > CH_2Cl_2 > CHCl_3 > CCl_4 > CBrCl_3$$

Recknagel et al. (1977) and Koch et al. (1974) demonstrated increased toxicity and a much greater increase in diene conjugation in microsomal lipids from rats treated with $CBrCl_3$ than with CCl_4. This correlates well with the bond dissociation energies of 49 and 68 kcal/mole for $CBrCl_3$ and CCl_4, respectively.

The events which mediate CCl_4 activation and cytotoxicity are complex, making it difficult to pinpoint the causes of cell necrosis. Some of the earlier effects of CCl_4 poisoning include the following: binding of CCl_4 to cellular lipids and proteins; increased peroxidation of microsomal lipids; destruction of cytochrome P_{450}, G6Pase, and subsequent loss of mixed-function

oxidase activity; polyribosome disaggregation; decreased synthesis of protein and phospholipid; triglyceride accumulation; and loss of NADPH, NADP, ascorbate, vitamin E, and glutathione (Halbreich and Mager, 1969; Recknagel, 1967; Recknagel and Glende, 1973; Slater, 1972). Damage to other organelles, such as mitochondria and lysosomes, occurs later in the course of CCl_4 poisoning, after evidence of cell necrosis has appeared (Recknagel, 1967).

There are two general hypotheses to account for CCl_4 toxicity: that proposed by Castro et al. (1972) which emphasizes the importance of binding to critical cell components, and that of Recknagel (1967) which emphasizes lipid peroxidation. Some support has been found for both hypotheses. Villarruel et al. (1977) and Diaz Gomez et al. (1975) investigated hepatotoxicity of CCl_4 in several animal species as well as general tissue damage within the same animal. Both studies demonstrated that tissue damage correlated better with binding to cellular components than with diene conjugation. Since arachidonic acid is destroyed during lipid peroxidation, Villarruel et al. (1976) also examined the effect of CCl_4 on the arachidonic acid content of microsomes from animals that showed moderate and high susceptibility to CCl_4-hepatotoxicity. Again, they were not able to correlate hepatotoxicity with lipid peroxidation. The species most susceptible to CCl_4-hepatotoxicity showed no change, whereas the species which was least susceptible showed significant changes in arachidonic acid content.

Recknagel (1967) and Slater (1976) developed the lipid peroxidation hypothesis to explain the widespread damage caused by CCl_4 within the cell. They suggested that binding hypothesis was unlikely due to the reactivity of $\cdot CCl_3$. It would be expected to have a very short biological half-life and would not migrate any significant distance from its site of formation in the microsomal membrane to damage other subcellular organelles. By-products of lipid peroxidation would be more stable and their toxic effects have been demonstrated (Dianzani et al., 1976; Gammage and Matsushita, 1973; Matsushita et al., 1970; Recknagel and Turocy, 1977; Ugazio et al., 1976).

The question as to which hypothesis is correct has not been resolved and remains an active area of investigation by several laboratories. What is certain is the fact that the metabolism of CCl_4 by the microsomal mixed-function oxidase system does initiate lipid peroxidation *in vitro* as well as *in vivo*. Due to my general interest in enzyme-catalyzed lipid peroxidation, a further investigation of some aspects of its metabolism and the relationship between the activation reactions and lipid peroxidation was undertaken.

The Peroxidation of Membrane Lipids

Biological membranes contain significant quantities of polyunsaturated fatty acids which are important for both their structural integrity and function (Slater, 1972). A common property of polyunsaturated fatty acids is their ability to undergo peroxidative destruction in the presence of oxygen and free radical

initiators. The reactions involved are complex but can be simplified into three general categories common to all free radical chain reactions (Pryor, 1976):

1. Initiation $\quad LH \xrightarrow{-H\cdot} L\cdot$

2. Propagation $\quad L\cdot + O_2 \longrightarrow LOO\cdot$

$\quad LOO\cdot + LH \longrightarrow LOOH + L\cdot$

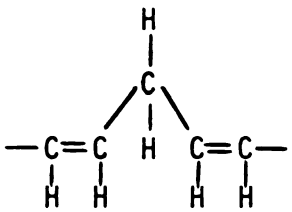
3. Termination $\quad 2\ L\cdot \longrightarrow L - L$

$\quad L\cdot + LOO\cdot \longrightarrow LOOL$

$\quad 2\ LOO\cdot \longrightarrow LOOL + {}^1O_2$

In biological systems the initial hydrogen abstraction to form the lipid radical, reaction (1), can be catalyzed by free radicals. These may be produced during either normal cell reactions or from the enzymatic activation of pro-oxidants, compounds whose metabolism or interaction with other compounds results in free radical formation (Plaa and Witschi, 1976). Another possibility involves the enzymatic reduction of ADP- or pyrophosphate-chelated iron and its subsequent reaction with oxygen to form the perferryl ion, a proposed initiator of lipid peroxidation (Pederson and Aust, 1975; Svingen et al., 1978).

Polyunsaturated fatty acids would be especially susceptible to attack because they all contain reactive hydrogens located on the methylene carbon shown below.



Hydrogen abstraction from this structure would result in the formation of resonance stabilized radicals. Reaction of these with hydrogen donors forms lipids containing conjugated dienes detectable by their absorption of light at 233 nm (Recknagel and Goshal, 1976). In the presence of oxygen, lipid hydroperoxides and more lipid radicals are formed as shown by reaction (2). The lipid hydroperoxides can be degraded by several cellular constituents with pro-oxidant activity including heme, hemoproteins, and transition metals which readily undergo one electron oxidation-reductions (O'Brien, 1961; O'Brien and Little, 1969; Wills, 1965). Products of this degradation are numerous and have yet to be thoroughly characterized for the more complex polyunsaturated fatty acids. Those which have been identified include lipid and alkoxy radicals, aldehydes (MDA), ketones, lipid alcohols, epoxides, and alkanes (Gardner, 1975; Gardner et al., 1974; Hamburg, 1974). Techniques for measuring lipid hydroperoxides directly or the degradation products, malondialdehyde, ethane, and pentane, are currently used to detect lipid peroxidation in vivo and in vitro (Dillard et al., 1976; Slater, 1972).

The formation of lipid hydroperoxides and their subsequent degradation by cellular pro-oxidants have important biological consequences. Since lipids are required for membrane integrity,

peroxidative destruction would disrupt membrane function and destroy many membrane-associated enzyme activities. Several degradation products of lipid hydroperoxides have been shown to be capable of crosslinking proteins, inhibiting several enzymes, causing red blood cell lysis, and killing microorganisms (Dianzani et al., 1976; Pfeifer and McCay, 1971; Recknagel and Turocy, 1977; Ugazio et al., 1976). Lipid hydroperoxides can also react directly with enzymes resulting in a loss of activity (Gamage and Matsushita, 1973; Matsushita et al., 1970).

Fortunately, under normal conditions, mechanisms exist which control the extent of lipid peroxidation. In addition to the termination process, reaction (3), the cell is protected to some extent by its relatively low oxygen content and the presence of both hydrophilic and lipophilic antioxidants (Recknagel, 1977). The reactions involved in propagation are believed to be blocked by many antioxidants which function as either radical trappers or alternate hydrogen donors. Radicals which result from the interaction with antioxidants are much less reactive and more likely to participate in termination reactions (Demopoulos, 1973; Mead, 1976). In addition, the cell contains enzymes with peroxidase activity which in the presence of suitable electron donors will reduce the lipid hydroperoxides to lipid alcohols and water. Two such enzymes involved in these reactions are glutathione peroxidase and cytochrome P_{450} (Hrycay and O'Brien, 1973; McCay, 1976; Sies and Summer, 1975).

CHAPTER II

THE ROLE OF THE NADPH-CYTOCHROME P_{450} REDUCTASE COMPONENT OF RAT LIVER MICROSOMES IN CARBON TETRACHLORIDE ACTIVATION

Abstract

A model membrane system was developed for determining the role of the flavoprotein, NADPH-cytochrome P_{450} reductase, in CCl_4 activation. The system contained purified cytochrome P_{450} reductase, liposomes, and NADPH in the presence or absence of the electron acceptors, ADP-Fe^{+3} and EDTA-Fe^{+3}. Conditions which favor the fully reduced and half-reduced flavoprotein could be achieved in the absence and presence, respectively, of the electron acceptors. The CCl_4-dependent stimulation of lipid peroxidation or the anaerobic-covalent binding of $^{14}CCl_4$ to liposomes was then used to assay CCl_4 activation by both forms of the enzyme.

The ability of the fully reduced NADPH-cytochrome P_{450} reductase to activate CCl_4 was investigated. CCl_4 addition to the liposomal system in the absence of electron acceptors did not cause the oxidation of NADPH. However, addition of EDTA-Fe^{+3} to the same system resulted in a substantial rate of NADPH oxidation. The fully reduced enzyme also did not cause the covalent binding of $^{14}CCl_4$ to liposomes.

To determine CCl_4 activation by the half-reduced NADPH-cytochrome P_{450} reductase both ADP-Fe^{+3} and EDTA-Fe^{+3} were included in the system. CCl_4-stimulated NADPH-dependent lipid peroxidation (MDA formation) and an NADPH-dependent binding of $^{14}CCl_4$ to liposomes were observed. Binding was also observed when either ascorbate or Fe^{+2} were added to the system in the absence of NADPH and NADPH-cytochrome P_{450} reductase. Activation by NADPH-cytochrome P_{450} reductase in the presence of NADPH, ADP-Fe^{+3}, and EDTA-Fe^{+3} was not observed unless the liposomes contained lipid hydroperoxides. The reductase was required to reduce EDTA-Fe^{+3} to its pro-oxidant form, EDTA-Fe^{+2}. The latter catalyzes the degradation of lipid hydroperoxides into lipid radicals which activate CCl_4 by atom transfer reactions. Cytochrome $P_{450}(Fe^{+3})$ as well as other oxidized hemoproteins will also serve as pro-oxidants in this system.

The results of these studies suggest that NADPH-cytochrome P_{450} reductase does not directly serve as a site of CCl_4 activation in the microsomal mixed-function oxidase system. Cytochrome P_{450} reductase participates primarily in CCl_4 activation through its reduction of cytochrome P_{450} and, alternatively, through its ability to generate lipid hydroperoxides in the presence of ADP-Fe^{+3} and oxygen. The hydroperoxides subsequently activate CCl_4 in the presence of cytochrome $P_{450}(Fe^{+3})$ or other cellular pro-oxidants.

Introduction

The metabolism of CCl_4 by the microsomal mixed-function oxidase system of the liver has been previously established (Recknagel et al., 1977). Unlike the majority of substrates for this system, the metabolism (activation) of CCl_4 can proceed by reductive reactions requiring NADPH but not oxygen (Glende et al., 1976; Uehleke et al., 1973). The reductive reactions involved and the role of each of the microsomal mixed-function oxidase components in CCl_4 activation have not been characterized. In other reductive reactions catalyzed by the mixed-function oxidase system, the azo- and nitro-reductases, both the cytochrome P_{450} and NADPH-cytochrome P_{450} reductase components will transfer reducing equivalents to the substrates (Gillette, 1971a). Evidence which suggests that these components function similarly in CCl_4 activation has been previously presented (Recknagel et al., 1977; Shah and Carlson, 1975; Slater, 1972). The evidence which supports the view of cytochrome P_{450} reductase being the locus of CCl_4 activation within the mixed-function oxidase system will be presented below.

Although a role for NADPH-cytochrome P_{450} reductase in CCl_4 activation has not been clearly established, it is believed to be the site of $\cdot CCl_3$ formation within the mixed-function oxidase system (Slater, 1971b). The general reactions by which this may occur, atom transfer and electron capture, were previously discussed. That NADPH-cytochrome P_{450} reductase also activates CCl_4 by one of these reactions is a possibility. Slater (1972) has proposed that an

electron capture reaction involving the fully reduced flavoprotein, NADPH-cytochrome P_{450} reductase, may be responsible for CCl_4 activation. An atom transfer reaction involving this enzyme is also possible. Masters et al. (1965) have shown that cytochrome P_{450} reductase shuttles between its fully and half-reduced states during the transfer of electrons to cytochrome P_{450} and other electron acceptors. In its half-reduced state, cytochrome P_{450} reductase contains the flavin semiquinone radical (Iyanagi et al., 1974) which may participate in CCl_4 activation directly or through formation of secondary radicals (Slater, 1974; Slater and Sawyer, 1971b).

Evidence which supports the hypothesis that NADPH-cytochrome P_{450} reductase is the locus of CCl_4 activation within the microsomal mixed-function oxidase system is as follows. Slater and Sawyer (1971a,b) used the CCl_4-dependent stimulation of microsomal lipid peroxidation, MDA production, as an indicator of $\cdot CCl_3$ formation. They found that compounds which interact with cytochrome P_{450} to inhibit mixed-function oxidase activity, e.g., SKF-525A, CO, and nicotinamide, did not decrease the CCl_4-dependent stimulation of lipid peroxidation. This was true even though these compounds caused a significant, 50 to 60%, inhibition of mixed-function oxidase activity, aminopyrine demethylation. Masuda and Murano (1977) developed a similar system but used EDTA-washed microsomes to greatly reduce endogenous rates of lipid peroxidation and to thereby increase the magnitude of CCl_4-stimulated lipid peroxidation. In their system CO inhibited aminopyrine demethylation by 91% but did

not decrease CCl_4-stimulated lipid peroxidation. Mixed-function oxidase substrates or inhibitors, aminopyrine and SKF-525A, were inhibitory only at relatively high concentrations where they are believed to function as antioxidants rather than competitive inhibitors. CCl_4-stimulated lipid peroxidation was completely inhibited, however, by low concentrations of substances which interact with cytochrome P_{450} reductase, pCMB, $K_3Fe(CN)_6$, cytochrome c, and vitamin K_3. This agreed with the earlier hypothesis which suggested that inhibition was due to the competition of these agents with CCl_4 for reducing equivalents from the fully reduced cytochrome P_{450} reductase (Slater and Sawyer, 1971b). It was also pointed out that the destruction of NADPH observed during CCl_4 intoxication was due to its interaction with $\cdot CCl_3$ at the activation site, NADPH-cytochrome P_{450} reductase (Slater and Sawyer, 1977).

Because of these findings an investigation was undertaken to assess the role of NADPH-cytochrome P_{450} reductase in CCl_4 activation. At the beginning of this investigation the objectives sought were to determine if the enzyme was capable of CCl_4 activation and, if so, to determine the reactions involved. A model membrane system consisting of liposomes prepared from extracted liver microsomal lipids, purified NADPH-cytochrome P_{450} reductase, and NADPH in the presence of absence of electron acceptors (ADP-Fe^{+3} and EDTA-Fe^{+3}) was used in these investigations. The stimulation of lipid peroxidation by CCl_4 and the binding of $^{14}CCl_4$ to liposomes was used to estimate $\cdot CCl_3$ formation.

Materials and Methods

Material Sources

Male Sprague-Dawley rats obtained from Spartan Research Animals, Haslett, Michigan, were used. Rats pretreated with PB and 3-MC ranged in weight from 225 to 250 g and from 90 to 125 g, respectively.

Soybean lipoxygenase (Type I), hemoglobin (Type IV), hemin (Type I), cytochrome c (Type VI), and bovine serum albumin (BSA) (Type V) were obtained from the Sigma Chemical Company, St. Louis, Missouri. Bromelain was a gift from the Dole Company, Honolulu, Hawaii.

Butylated hydroxytoluene (BHT), dithiothreitol (reagent grade), sodium cholate, Sepharose 4-B, sodium dodecyl sulfate (SDS), thiobarbituric acid, Tris base, ADP (Grade III), NADPH, Dowex chelating resin, 1,4-bis [2-(5 phenyloxazolyl)] benzene (POPOP), 3-methylcholanthrene (3-MC), polyethyleneglycol 400, and Brilliant Blue R (Coomassie blue) were obtained from the Sigma Chemical Company, St. Louis, Missouri. Glass-distilled toluene was obtained from Burdick and Jackson Lab., Inc., Muskegon, Michigan. 2,5-Diphenyl oxazole (PPO) was obtained from Research Products International, Elk Grove, Illinois. Deoxycholic acid, 1,8-diamino octane, cyanogen bromide, and anthraquinone 2-sulfonic acid were obtained from the Aldrich Chemical Company, Milwaukee, Wisconsin. EDTA and sodium hydrosulfite (dithionite) were obtained from the Fisher Scientific Company, Fairlawn, New Jersey. All electrophoresis reagents were from Canalco, Inc., Rockville, Maryland. Sucrose was from the Schwartz-Mann Division of Becton-Dickinson and Co., Orangeburg,

New York. Emulgen 913 was a gift from Kao-Atlas, Ltd., Tokyo, Japan. Phenobarbital (PB) was from Merck & Co., Inc., Rahway, New Jersey. $^{14}CCl_4$ and [^{14}C] toluene were from Amersham Searle, Arlington Heights, Illinois. $^{14}CCl_4$ was also obtained from New England Nuclear, Boston, Massachusetts. Dilauroylglyceryl-3-phosphoryl choline (di-1,2-GPC) was obtained from Cal-Biochem, San Diego, California. 2',5'-ADP-Sepharose 4B was obtained from P. L. Biochemicals, Milwaukee, Wisconsin. Hydroxyapatite (Biogel-HTP), calcium phosphate gel, and Biobeads SM2 were obtained from Biorad, Richmond, California. Argon and nitrogen gases (99.99% purity) and oxygen (99.5% purity) were obtained through General Stores, Michigan State University, East Lansing, Michigan. CO (PCP grade, 99.5% purity) was obtained from Matheson, E. Rutherford, New Jersey. All other reagents used were of analytical grade.

All aqueous solutions were prepared from water which had been distilled and passed through a mixed-bed ion exchange column. Cholic and deoxycholic acids were treated with activated charcoal and recrystallized from 95% ethanol prior to use.

Drug Pretreatment of Rats

Rats were treated with PB by including it in their drinking water at a concentration of 0.1% for 10 days. Rats were treated with 3-MC by i.p. injection of a dose (20 mg/kg, dissolved 10 mg/ml in polyethyleneglycol-400) at 36 and 24 hours prior to sacrifice.

Isolation of Rat Liver Microsomes

Rats were fasted 18 hours prior to killing by decapitation. Microsomes were prepared by the differential centrifugation

procedure of Pederson (1973) except as modified below. Microsomes to be used for the activation of $^{14}CCl_4$ were washed by resuspending in 1 mM Tris-HCl (pH 7.5 at 25°C) to a final protein concentration of from 3 to 5 mg/ml and recentrifuging as described. Microsomes were stored in argon-saturated 0.05 M Tris-HCl (pH 7.5 at 25°C) containing 50% glycerol as described but BHT was omitted. Stock buffers of Tris-HCl were treated with Dowex chelating resin to remove trace amounts of iron. These "chelexed" buffers were subsequently used to prepare the Tris buffers mentioned above. Microsomes isolated in this manner were used within two days.

Microsomes used for cytochrome P_{450} and P_{448} isolations were prepared as above but were isolated and washed in 1.15% KCl containing 10 mM EDTA and stored in the presence of 0.01% BHT. Microsomes used for the preparation of NADPH-cytochrome P_{450} reductase were isolated and stored in the presence of BHT according to Pederson (1973) but were washed prior to use according to Yasukochi and Masters (1976). All microsomal preparations were stored under argon at -20°C.

Purification of NADPH-Cytochrome P_{450} Reductase from Rat Liver Microsomes

Two procedures were used to isolate cytochrome P_{450} reductase from the microsomes from PB pretreated rats. The procedure of Dignam and Strobel (1975) was used without modification; that of Yasukochi and Masters (1976), with the modifications included below.

Gel filtration on LKB ultrogel AcA 34 was omitted and treatment with Biobeads SM2 for 90 minutes at 0-4°C (Holloway, 1973) was used for detergent removal instead of DEAE-cellulose chromatography.

Purification of Cytochromes P_{450}, P_{448}, and b_5 from Rat Liver Microsomes

A column (2.6 x 40 cm) of 8-aminooctyl Sepharose 4B was prepared according to the procedures of Imai and Sato (1974) and Cuatrecasas (1970). This column was subsequently used to purify cytochromes P_{450}, P_{448}, and b_5 according to the combined procedures of Imai (1976) and Guengerich (1977). The latter method was used to purify cytochromes P_{450} or P_{448} from the microsomes of PB- or 3-MC-pretreated rats, respectively. Modifications of the procedure are included below. All buffers were adjusted to the proper pH at room temperature prior to the addition of dithiothreitol. The deionized-distilled water used to prepare buffers for hydroxyapatite chromatography was degassed to remove CO_2 traces and help maintain good flow rates. Biobeads SM2 treatment (90 minutes, 0-4°C) followed by adsorption, washing, and elution from calcium phosphate gel, was used to remove excess detergent, Emulgen 913. The DEAE-cellulose chromatography was omitted because it did not improve the specific content and resulted in even lower yields of cytochrome P_{450} (P_{448}). Peak fractions which eluted from the hydroxyapatite column with 90 and 150 mM "phosphate buffers" were pooled and concentrated four to six-fold by ultrafiltration on an Amicon

PM30 membrane. Detergent was removed as described and the fractions were dialyzed against 30 volumes of 10 mM Tris-acetate (pH 7.4 at 25°C) containing 20% glycerol and 0.1 mM EDTA. The dialyzed fractions were centrifuged at 20,000 x g or higher for 20 minutes to remove any precipitate and stored under argon at -20°C. If a substantial precipitate forms, as has happened in the purification of cytochrome P_{448}, a 0.1 M potassium phosphate buffer, pH 7.25, containing 20% glycerol, 0.1 mM EDTA, and from 0.01 to 0.05% Emulgen 913 can be used for redissolving and storage. The P_{448} remained stable and could be used in experiments in which detergent contamination would not interfere.

Cytochrome b_5 was prepared by subsequent elution from the octylamine column by the method of Imai (1976). The column was first washed with 0.05 M potassium phosphate buffer (pH 7.25) containing 20% glycerol, 0.15% deoxycholate, and 0.35% cholate (approximately 1 to 1.5 liters) to remove an NADPH-cytochrome P_{450} reductase-containing fraction with specific activity ranging from 10 to 15 units/mg protein. Cytochrome b_5 was eluted with a 0.05 M potassium phosphate buffer (pH 7.25) containing 20% glycerol, 0.35% deoxycholate and 0.15% cholate. Peak fractions were pooled, concentrated by ultrafiltration, treated with Biobeads SM2, dialyzed as previously mentioned, and stored. The detergents deoxycholate and cholate were still contaminating this preparation and were not significantly decreased by further Biobead treatment or dialysis for 2 days against the "Tris-acetate" buffer. Unless otherwise

stated, all hemoprotein preparations were run over a Sephadex G25 column, equilibrated in 0.1 M Tris-HCl (pH 7.25 at 25°C and chelexed) buffer containing 20% glycerol, before being used in experiments described in this thesis.

When required the octylamine column was regenerated by passing approximately five column volumes of 0.1 M potassium phosphate buffer (pH 7.25) containing 20% glycerol, 0.2% Emulgen 913, 0.6% cholate, and 1 mM EDTA. The column was then washed with three column volumes of the equilibration buffer as described.

Enzyme Assays and Analytical Procedures

Cytochrome P_{450}, P_{448}, and b_5 (Omura and Sato, 1964) and NADPH-cytochrome c reductase (Pederson, 1973) were assayed as previously described. Hemoglobin was treated with potassium ferricyanide as described (Antonini and Brunori, 1971), and subjected to column chromatography on Sephadex G25. The concentration of the methemoglobin formed was determined spectrally in 0.1 M potassium phosphate buffer (pH 6.5) using an extinction coefficient of 179 $mM^{-1}cm^{-1}$ for the peak at 405 nm (Winterhalter, 1974). NADH-cytochrome b_5 reductase activity was measured by determining the reduction of $K_3Fe(CN)_6$. The 1 ml assay contained 0.1 M potassium phosphate buffer (pH 7.4), 1 mM $K_3Fe(CN)_6$, 0.25 mM NADH, and from 0.02 to 0.4 mg protein. The change in absorbance at 420 nm was followed with time and an extinction coefficient of 1.02 $mM^{-1}cm^{-1}$ used for reduced ferricyanide. All assays were performed at 25°C unless stated otherwise.

The activation of $^{14}CCl_4$ was determined by measuring its covalent binding to lipid. The 2 ml liposomal and microsomal assay systems differed in their initial preparation but not extraction and subsequent analytical procedures. All reactions were run under anaerobic conditions, which were established as follows: all buffers and reagents were thoroughly saturated with argon by bubbling for approximately 30 min (0-4°C) prior to use; microsomes or other reaction components were added to 10 ml erlenmeyers under a stream of argon and capped with gas-tight rubber caps (either caps from Vac-u-tainer blood-collecting tubes or serum caps were used); and each flask was evacuated and flushed five times with de-oxygenated argon and left under slightly positive argon pressure. $^{14}CCl_4$ and reagents required for the initiation of the reactions (NADPH, $Fe(NH_4)_2(SO)_2$, ascorbate, or partially peroxidized lipid) were added via glass syringe. All of the above procedures were performed at 0-4°C. To conduct the reactions, flasks were preincubated for 3 min (at 37°C) and the reaction initiated and run for a designated period of time with constant shaking (90 to 100 rpm). Reactions were stopped with the addition, via glass syringe, of 2 ml of cold nitrogen-saturated chloroform-methanol ($CHCl_3$:MeOH) (1:1 v/v) containing 0.01% BHT and placed on ice with caps in place. When all reactions were completed the flasks were allowed to reach room temperature before extraction proceeded. The contents of each flask were quantitatively transferred to 20 ml screw-cap culture tubes, 2.4 ml $CHCl_3$:MeOH (0.01% BHT) was then used to rinse each

flask and added to the previous fraction to achieve a final $CHCl_3$: MeOH:H_2O ratio of 2:2:1.8 used in the lipid extraction procedure of Bligh and Dyer (1959). The tubes were capped, vortexed for 50 sec, and centrifuged. After discarding the aqueous phase, 0.2 volumes of Folch-salts (Folch et al., 1956) were added to the 2.4 ml organic phase and the procedure repeated. The washed organic phase was quantitatively transferred to tared scintillation vials and evaporated to dryness at 45°C under a stream of nitrogen. To the residue were added successively 0.2 and 0.1 ml aliquots of CCl_4 and absolute ethanol, respectively, and the mixture taken to dryness after each addition. Reactions were conducted and extractions performed under subdued lighting conditions, whenever possible. The weights of the lipid residues were determined and prepared for scintillation counting.

To prepare the extracted lipid for scintillation counting, 15 ml of toluene containing 0.5% PPO and 0.03% POPOP were added to the scintillation vials. The optimum instrumental conditions were determined for counting ^{14}C and where required were quench-corrected by the Channels Ratio method (Bush, 1963). Reactions were generally performed in triplicate and the Student t-test used to determine significance of differences between the means (Klugh, 1974).

CCl_4 activation was also measured by its ability to enhance lipid peroxidation. For these reactions lipid and hemoproteins were mixed first, 10 min at 0-4°C, prior to addition of other reagents. Other components were then added as described in each figure legend,

at 0-4°C. The mixture was subsequently preincubated for 2 min at 37°C prior to initiation of the reaction. MDA was determined from 1-ml aliquots as described elsewhere. Only the protease-solubilized NADPH-cytochrome P_{450} reductase was used for these experiments. Unless otherwise stated, ADP-Fe^{+3} and EDTA-Fe^{+3} were previously mixed in 0.05 M Tris HCl and pH adjusted to 7.5 at 25°C; their respective molar ratios to iron were 16.6:1 and 1:1. Buffers treated with Dowex chelating resin were used for all reactions in which CCl_4 activation was determined.

Malondialdehyde (MDA) and lipid hydroperoxides (Buege and Aust, 1978) and total lipid phosphate (Bartlett, 1959) were determined as previously reported. Protein was determined by the method of Lowry et al. (1951) and standardized with bovine serum albumin using $E^{1\%}_{cm}$ at 280 nm equal to 6.6 (Rutter, 1967).

SDS-Polyacrylamide Gel Electrophoresis

Electrophoresis was carried out according to the procedure of Welton and Aust (1973) with the following modifications: samples were dialyzed overnight against 50 volumes of distilled-deionized water and lyopholyzed; and 1.5% SDS was included in the prepared sample to ensure migration of all protein into the gel matrix.

Extraction of Lipids and the Preparation of Liposomes

The procedures of Pederson (1973) were used for the extraction of lipid from the microsomes of untreated rats and for the preparation of liposomes. Liposomes were prepared in

argon-saturated deionized-distilled water. When synthetic lipid was used a small quantity was weighed, distilled water added, and sonicated as before.

Preparation of Lipid Hydroperoxides

Soybean lipoxygenase was used to generate lipid hydroperoxides in a reaction containing 0.05 M borate buffer (pH 9.0), 0. 1 mM EDTA, 0.3% sodium deoxycholate, 0.10 mg lipoxygenase/ml, and from 2.5 to 5.0 μmole lipid-PO_4/ml of reaction. Reactions, 20 ml, were carried out in 250 ml erlenmyer flasks under a stream of oxygen with constant agitation. The reaction was stopped after 30 min by the addition of $CHCl_3$:MeOH (1:2) to form a single phase consisting of $CHCl_3$:MeOH:H_2O in a ratio of 1:2:0.8. A sufficient volume of $CHCl_3$:H_2O (1:1) was subsequently added to yield $CHCl_3$:MeOH:H_2O ratios of 2:2:1.8 as required by Bligh and Dyer(1959) for total lipid extraction. The resulting mixture is centrifuged and the organic phase washed with 0.2 volume Folch-salts solution (Folch et al., 1956). The organic phase was concentrated by evaporation under a stream of nitrogen at 45°C and stored under argon at -20°C. Storage under these conditions resulted in little or no loss of hydroperoxides for periods up to 5 days.

Deoxygenation of Argon

Argon was used as the gas phase in all of the reactions conducted anaerobically. Deoxygenation was according to the method of Vogel (1958) with a saturated solution of lead acetate included

in the line to trap any H_2S which may be evolved. Thick-walled rubber tubing was used thought to minimize oxygen leakage or diffusion into the system (Dixon, 1971a).

Results

Purification of NADPH-Cytochrome P_{450} Reductase from Rat Liver Microsomes

In order to determine the role of cytochrome P_{450} reductase in CCl_4 activation, the pure enzyme is required. During the course of these investigations two purification procedures were used, that of Dignam and Strobel (1975) and of Yasukochi and Masters (1976). An excellent preparation resulted from both procedures; however, since the latter preparation was used in the majority of these studies, only data concerning its purification will be presented. Table 1 summarizes the purification of NADPH-cytochrome P_{450} reductase from liver microsomes of PB-pretreated rats. The specific activity obtained, 47.7 μmoles cytochrome c reduced/min/mg protein, was comparable to that reported when measured under their assay conditions (same as Methods section of this chapter but at 30°C). The peak fractions eluting from the 2',5'-ADP-sepharose affinity column were sufficiently pure and therefore the final gel filtration step on Ultrogel AcA-34 was omitted. The purified reductase contained a minor contaminant as shown in Figure 1.

TABLE 1.--The Purification of NADPH-Cytochrome P_{450} Reductase from Rat Liver Microsomes.

Fraction	Protein (mg)	Total Units*	Spec. Act. (units/mg)	Yield (%)
Washed PB-microsomes	1,600	705.6	0.44	100
DEAE column (peak fractions)	80	314.2	3.93	45
2',5'-ADP affinity column (peak fractions after Sephadex G25 treatment)	--	--	24.36	--
Affinity column fractions after Biobeads SM2 treatment and dialysis	4.5	164.2	36.50**	23

*1 unit = 1 μmole cytochrome c reduced/min when assayed as described in Methods.
**47.7 units/mg when assayed at 30°C.

Activation of CCl_4 by NADPH-Cytochrome P_{450} Reductase in Liposomes

Slater (1972) has proposed a reaction for the activation of CCl_4 by NADPH-cytochrome P_{450} reductase involving electron capture from the fully reduced flavoprotein. Two approaches were used to investigate this possibility. The first involved a determination of NADPH oxidation in a model membrane system containing only NADPH-cytochrome P_{450} reductase and liposomes prepared from the saturated lipid, di-1,2-GPC. Table 2 contains the results of this investigation. NADPH oxidation, as determined by the decrease in absorbance at 340 nm, was not observed in the presence of CCl_4. However, the addition of EDTA-Fe^{+3} (1:1 molar ratio), a known

Figure 1.--SDS-Polyacrylamide Gel Electrophoresis of NADPH-Cytochrome P_{450} Reductase Purified from PB-Microsomes.

NADPH-cytochrome P_{450} reductase was purified as described in Methods. A sample containing 10 µg of protein was applied to the gel and electrophoresis conducted in the presence of 1% SDS as described in Methods. The gel was stained for protein with Coomassie blue and photographed.

Fig. 1

TABLE 2.--NADPH-Cytochrome P_{450} Reductase Catalyzed Oxidation of NADPH in Liposomes Using CCl_4 and EDTA-Fe^{+3} as Electron Acceptors.

Reactions contained 0.1 mg di-1,2-GPC/ml, 0.025 units/ml NADPH-cytochrome P_{450} reductase, ± 0.2 mM EDTA with 0.2 mM $FeCl_3$, ± 1 µl CCl_4/ml, and 0.1 mM NADPH in 0.1 M Tris-HCl, pH 7.4 at 37°C. The reactions were conducted at 37°C and OD_{340} measured.

Description	nmole NADPH Oxidized/min/ml
Complete system	0
Complete system + CCl_4	0
Complete system + CCl_4 + EDTA-Fe^{+3}	1.70

electron acceptor (Noguchi and Nakano, 1974), resulted in a significant rate of NADPH oxidation. These results suggest that CCl_4 is not a good electron acceptor and hence not activated significantly under these experimental conditions. A more sensitive and direct assay was used to exclude the possibility that CCl_4 was being activated at rates too low to be measured by following NADPH oxidation. The covalent binding of $^{14}CCl_4$ to liposomes under anaerobic conditions was used to indicate CCl_4 activation. Anaerobic conditions were used to exclude the interference of oxygen with binding (Uehleke et al., 1973; Wood et al., 1976) and to exclude the possibility of its competing with CCl_4 for electrons from the fully reduced enzyme. Oxygen is capable of accepting electrons from the fully reduced enzyme and would be expected to decrease its steady-state levels (Dixon, 1971b). The results of Table 3 confirm the findings of the previous experiment suggesting that the

TABLE 3.--NADPH-Cytochrome P_{450} Reductase Catalyzed Activation of $^{14}CCl_4$ in Liposomes in the Absence of Added Electron Acceptors.

Reactions contained 1.0 μmole lipid-PO_4/ml, 0.01% sodium deoxycholate, 1 μl $^{14}CCl_4$/ml (0.25 m Ci/ml CCl_4) ± 0.3 mM NADPH in 0.1 M Tris-HCl, pH 7.4 at 37°C. The reactions were conducted under anaerobic conditions as described in Methods for 30 min at 37°C.

Description	cpm/mg Lipid ± S.D.
Liposomes (-) NADPH (-) NADPH cytochrome P_{450} reductase	515 ± 10
Liposomes + 0.3 mM NADPH + 0.025 units reductase/ml	496 ± 28
Liposomes + 0.3 mM NADPH + 0.100 units reductase/ml	529 ± 73

hypothesis of CCl_4 activation involving electron capture from the fully reduced enzyme is incorrect. The experimental conditions favored the full reduction of NADPH-cytochrome P_{450} reductase; however, CCl_4 was not activated at either enzyme concentration used.

CCl_4 Activation by NADPH-Cytochrome P_{450} Reductase in the Presence of Added Electron Acceptors

A property of NADPH-cytochrome P_{450} reductase which has led to its proposed function in CCl_4 activation is its ability to form semiquinone radicals during its electron transfer reactions (Iyanagi et al., 1974). A plausible mechanism for CCl_4 activation would involve atom transfer from either the flavin semiquinone radical or perhaps secondary radicals generated as a result of its

formation. One suitable system in which to investigate CCl_4 activation via the half-reduced enzyme would be that developed by Noguchi and Nakano (1974) and Pederson and Aust (1973) for the characterization of NADPH-cytochrome P_{450} reductase-dependent lipid peroxidation. This well-characterized system contains NADPH-cytochrome P_{450} reductase, liposomes, and ADP-Fe^{+3} and EDTA-Fe^{+3}, as electron acceptors. The formation of $\cdot CCl_3$ can be estimated from the stimulation of lipid peroxidation, MDA formation. Table 4 presents the initial experiments to demonstrate CCl_4 activation by this system. A small but reproducible stimulation of MDA production resulted from the addition of CCl_4. To increase the sensitivity of this assay, the anaerobic binding of $^{14}CCl_4$ to liposomes, prepared from extracted microsomal lipid, was again utilized as an indicator of CCl_4 activation. These experiments resulted in a small but

TABLE 4.--CCl_4-Stimulated Lipid Peroxidation in Liposomes.

Reactions contained 0.5 μmole lipid-PO_4/ml, 0.02 units/ml NADPH-cytochrome P_{450} reductase, 1.66 mM ADP with 0.1 mM $FeCl_3$, 0.05 mM EDTA, 0.1 M NaCl, 1 μl CCl_4/ml ± 0.25 mM NADPH in 0.1 M Tris-HCl, pH 7.4 at 37°C. Reactions were carried out for 15 min at 37°C and MDA determined as described in Methods.

Description	nmoles MDA/15 min/ml
Liposomes (-) NADPH	0.12
Liposomes (+) NADPH	3.80
Liposomes (+) NADPH (+) CCl_4	4.58

statistically significant increase in labeling in the presence of NADPH (Table 5).

TABLE 5.--NADPH-Cytochrome P_{450} Reductase Catalyzed Activation of $^{14}CCl_4$ in Liposomes in the Presence of Electron Acceptors.

Reactions contained 1.0 μmole lipid-PO_4/ml, 0.02 unit/ml NADPH-cytochrome P_{450} reductase, 1.66 mM ADP with 0.1 mM $FeCl_3$, 0.05 mM EDTA, 1 μl $^{14}CCl_4$/ml (0.25 m Ci/ml CCl_4), ± 0.3 mM NADPH in 0.1 M Tris-HCl, pH 7.4 at 37°C. Reactions were conducted under anaerobic conditions as described in Methods for 30 min at 37°C.

Description	cpm/mg Lipid ± S.D.	Expt'l.-Cont.
Liposomes (-) NADPH	484 ± 36	
Liposomes (+) NADPH	621 ± 54	137*

*Significantly different from control, $p < 0.05$.

It was of interest to determine the fate of cytochrome P_{450} reductase during activation by this system. If the mechanism of activation involves an atom transfer reaction between CCl_4 and the flavin semiquinone radical, one should expect chlorination of the flavin moiety and perhaps inactivation of the enzyme. That inactivation does not occur is indicated by the results presented in Table 6. Using similar reaction components and assay conditions as in the previous experiment, cytochrome c was added to these incubations at the end of 30 minutes and tested for enzymatic activity, the reduction of cytochrome c. In neither of these reactions was the reduction of cytochrome c by the enzyme decreased. The presence of oxygen (and hence lipid peroxidation) also did not alter enzymatic activity. These results are in agreement with the

TABLE 6.--The Fate of NADPH-Cytochrome P_{450} Reductase During CCl_4 Activation in Liposomes.

Reactions contained 0.1 μmole lipid-PO_4/ml, 0.018 units/ml NADPH-cytochrome P_{450} reductase, 1.66 mM ADP with 0.1 mM $FeCl_3$, 0.05 mM EDTA, 1 μl CCl_4/ml, ± 0.1 mM NADPH in 0.1 M Tris-HCl, pH 7.4 at 37°C. Reactions were conducted as follows: the 0.8 ml reaction mixture was incubated under anaerobic or aerobic (99% O_2) conditions for 30 min at 37°C after which it was placed on ice. Reactions were allowed to reach room temperature and NADPH and cytochrome c added to final concentrations of 0.2 mM and 75 μM, respectively. Cytochrome P_{450} reductase activity was determined according to Methods section.

Description		nmoles Cytochrome c Reduced/min/ml
Experiment 1 (anaerobic conditions)		
Complete system	(-) NADPH	3.6
	(+) NADPH	4.1
	(+) NADPH + CCl_4	4.4
Experiment 2 (aerobic conditions)		
Complete system	(-) NADPH	4.6
	(+) NADPH	6.1
	(+) NADPH + CCl_4	7.0

observations that reductase is not destroyed in CCl_4-poisoned animals or during the *in vitro* metabolism of CCl_4 by microsomes (Glende et al., 1976; Recknagel and Glende, 1973).

Determination of Optimal NADPH-Cytochrome P_{450} Reductase and EDTA-Fe^{+3} Concentrations for CCl_4 Activation in Liposomes

Having established that CCl_4 activation does occur in the liposomal system, a determination of the optimal experimental

conditions was pursued. If the flavin semiquinone of NADPH-cytochrome P_{450} reductase participates in CCl_4 activation, binding should increase with the enzyme content of the assay. The results of an experiment in which the dependence of activation on NADPH-cytochrome P_{450} reductase concentrations were determined are shown in Table 7. A correlation between activation and reductase content of the assay was not observed in this experiment in which EDTA-Fe^{+3} alone was included as the electron acceptor. The same was also true when both ADP-Fe^{+3} and EDTA-Fe^{+3} were added as electron acceptors (data not included). It therefore became apparent that a factor other than NADPH-cytochrome P_{450} reductase was limiting.

The concentration of EDTA-Fe^{+3} used for the above experiment is lower than that found optimal for the NADPH-cytochrome P_{450}

TABLE 7.--The Dependence of $^{14}CCl_4$ Activation on NADPH-Cytochrome P_{450} Reductase Concentration in Liposomes.

Reactions contained 1.0 µmole lipid-PO_4/ml, 0.1 mM EDTA with 0.1 mM $FeCl_3$, 0.25 M NaCl, 0.05% sodium deoxycholate, 1 µl $^{14}CCl_4$/ml (0.25 m Ci/ml CCl_4), 0.3 mM NADPH in 0.1 M Tris-Hcl, pH 7.4 at 37°C. The reactions were conducted under anaerobic conditions as described in Methods for 30 min at 37°C.

Description		cpm/mg Lipid ± S.D.
Liposomal System		
0 unit/ml	NADPH-cytochrome P_{450} reductase	1,638 ± 110
0.005 unit/ml	NADPH-cytochrome P_{450} reductase	1,528 ± 129
0.020 unit/ml	NADPH-cytochrome P_{450} reductase	1,557 ± 28
0.100 unit/ml	NADPH-cytochrome P_{450} reductase	1,521 ± 143

reductase-dependent lipid peroxidation in liposomes. Of the electron acceptors present, EDTA-Fe^{+3} is reduced most readily by the enzyme (Noguchi and Nakano, 1974); its increase would also increase the number of enzyme molecules in its half-reduced, flavin semiquinone form. The results of the experiment to determine the effect of EDTA-Fe^{+3} on CCl_4 activation is presented in Figure 2. A substantial increase in activation was obtained at EDTA-Fe^{+3} levels ranging from 0.05 to 0.5 mM. The relationship between CCl_4 activation and EDTA-Fe^{+3} concentration suggests a role for the flavin semiquinone radical of NADPH-cytochrome P_{450} reductase in CCl_4 activation; however, alternative interpretations are possible. As mentioned in the Literature Review of this thesis, some transition metals, including iron, are oxidized in the presence of haloalkanes resulting in free radical formation (Asscher and Vofsi, 1963; Castro and Kray, 1966). Though these reactions required non-physiological conditions, chelation by EDTA could sufficiently alter the properties of iron to make this a plausible mechanism. Another possibility stems from the fact that the reduced chelate, EDTA-Fe^{+2}, functions as a pro-oxidant catalyzing the degradation of lipid hydroperoxides (Svingen et al., 1978). Since free radicals are included among these products, CCl_4 may be subsequently activated through atom transfer reactions (O'Brien, 1969; O'Brien and Little, 1969). Although care was taken to exclude O_2 from both the liposomal preparation as well as the assays themselves, one cannot exclude completely the presence of some endogenous lipid

Figure 2.--The Effect of EDTA-Fe^{+3} on $^{14}CCl_4$ Activation in Liposomes.

Reactions contained 1.0 µmole lipid-PO_4/ml, 0.02 unit/ml NADPH-cytochrome P_{450} reductase, 0 to 1.0 mM EDTA-Fe^{+3} (1:1 mole ratio), 1.66 mM ADP-Fe^{+3} with 0.1 mM $FeCl_3$, 1 µl $^{14}CCl_4$/ml (0.25 m Ci/ml CCl_4), ± 0.3 mM NADPH in 0.1 M Tris-HCl, pH 7.4 at 37°C. Reactions were conducted under anaerobic conditions as described in Methods for 30 min at 37°C.

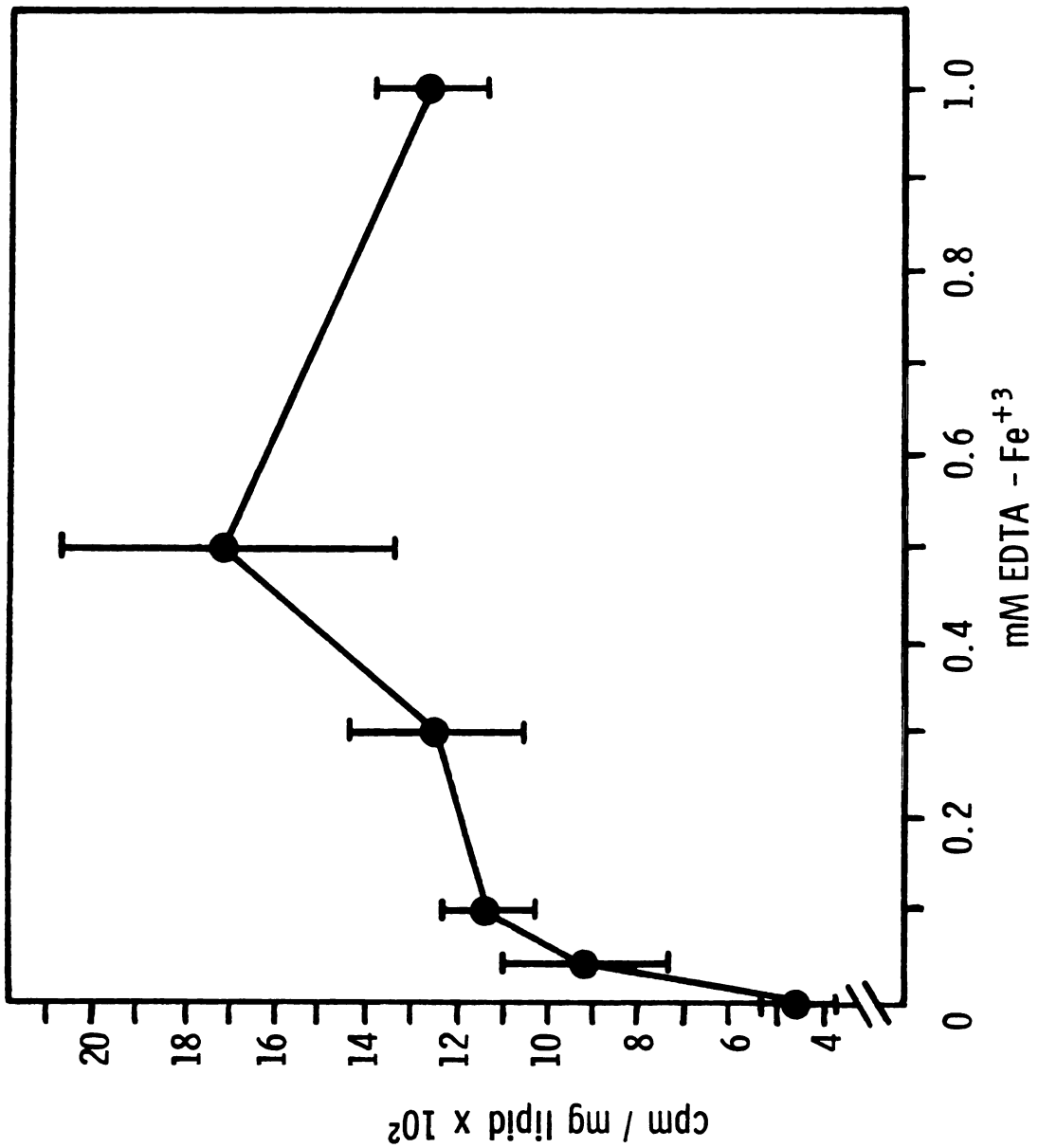


Fig. 2.

hydroperoxides. They may either have been present in the extracted microsomal lipid or result from ADP-Fe^{+3}-dependent initiation in the presence of trace amounts of oxygen in the system. Leakage or introduction along with the cofactors used to initiate the reactions may have been the source of this oxygen. Investigations to determine which of the above hypotheses is correct are presented in the following section.

Activation of CCl_4 in Liposomes Containing Ascorbate as the Source of Reducing Equivalents

If the pro-oxidant activity of EDTA-Fe^{+2} is solely responsible for increases in CCl_4 activation, sources of reducing equivalents other than NADPH and NADPH-cytochrome P_{450} reductase should work as well. Ascorbate will reduce chelated forms of iron and initiate lipid peroxidation *in vitro* and was used in these experiments (Pederson and Aust, 1973; Slater, 1972). For the sake of comparison, an ascorbate concentration was chosen that catalyzed similar rates of lipid peroxidation as the aerobic liposomal system containing NADPH-cytochrome P_{450} reductase. Both the ascorbate system and that to which Fe was added directly were shown capable of CCl_4 activation. Greater binding was observed in the ascorbate-containing system than that in the enzymatic system (Table 8). The difference is probably due to additional activation by the ascorbyl radical intermediate formed during the reduction of chelated iron (Slater, 1972).

TABLE 8.--Ascorbate and Fe^{+2} Catalyzed Activation of $^{14}CCl_4$ in Liposomes.

Reactions contained 1.0 µmole lipid-PO_4/ml, 0.05 mM EDTA, either 0.1 mM ascorbate + 1.66 mM ADP ± 0.1 mM Fe^{+3} or 1.66 mM ADP and from 0 to 5.0 mM Fe^{+2}, 1 µl $^{14}CCl_4$/ml (0.25 m Ci/ml CCl_4) in 0.1 M Tris-HCl, pH 7.4 at 37°C. Reactions were conducted under anaerobic conditions as described in Methods for 30 min at 37°C.

Description	cpm/mg Lipid ± S.D.	Expt'l.-Cont.
Liposome-ascorbate system		
(-) Fe^{+3}	440 ± 11	--
(+) 0.1 mM Fe^{+3}	799 ± 65	359*
Liposome-Fe^{+2} system		
(-) Fe^{+2}	390 ± 10	--
(+) 0.5 mM Fe^{+2}	599 ± 76	209*
(+) 1.0 mM Fe^{+2}	692 ± 56	302*
(+) 2.0 mM Fe^{+2}	928 ± 21	538*
(+) 5.0 mM Fe^{+2}	1,005 ± 35	615*

*Significantly different from control, $p < 0.05$.

The Role of Lipid Hydroperoxides in CCl_4 Activation by the Liposomal System

Activation of CCl_4 has been found to be dependent on the concentration of EDTA-Fe^{+2}. This form of iron may activate by either the pro-oxidant mechanism or by passing electrons directly to CCl_4. To distinguish between these possibilities, binding experiments were conducted in the presence and absence of liposomes

containing lipid hydroperoxides. Liposomes from freshly prepared lipid, containing no detectable lipid hydroperoxide content, and from lipoxygenase-treated lipid with 0.096 μmole LOOH/μmole lipid-PO_4 were used. Table 9 shows the results of an experiment which compares the activation of CCl_4 in assays containing the fresh and partially peroxidized liposomes. Significant activation was observed only in the presence of lipid hydroperoxides. Background activation was noticeably higher in partially peroxidized liposomes, probably being due to the degradation of lipid hydroperoxides not dependent on the pro-oxidant, EDTA-Fe^{+2}. The cause of the background labeling observed in fresh liposomes is still uncertain but may be due to levels of lipid hydroperoxides not detected by the

TABLE 9.--Dependence of $^{14}CCl_4$ Activation on the Lipid Hydroperoxide Contents of Liposomes.

Reactions contained 1.0 μmole unperoxidized or partially peroxidized lipid-PO_4/ml (0.096 μmole LOOH/μmole lipid-PO_4), 0.02 unit/ml NADPH-cytochrome P_{450} reductase, 1.66 mM ADP with 0.1 mM $FeCl_3$, 0.5 mM EDTA with 0.5 mM $FeCl_3$, 0.3 M NaCl, 0.05% sodium deoxycholate, 1 μl $^{14}CCl_4$/ml (0.25 m Ci/ml CCl_4), ± 0.3 mM NADPH in 0.1 M Tris-HCl, pH 7.4 at 37°C. Reactions were conducted under anaerobic conditions as described in Methods for 30 min at 37°C.

Description	cpm/mg Lipid ± S.D.	Expt'l.-Cont.
Unperoxidized Liposomal System		
(-) NADPH	160 ± 32	--
(+) NADPH	172 ± 14	12
Partially Peroxidized Liposomal System		
(-) NADPH	272 ± 48	--
(+) NADPH	545 ± 77	273*

*Significantly different from control, $p < 0.01$.

assay procedure used. As a precaution, lipid was routinely extracted from the reactions with nitrogen-saturated $CHCl_3$:MeOH which contained the antioxidant, butylated hydroxy toluene, 0.01%. This should minimize further peroxidation and subsequent labeling after the reaction has been stopped. It is also unlikely that extracted lipid residue contained some trapped non-covalently bound $^{14}CCl_4$. The lipid extract was evaporated to dryness under a stream of nitrogen and the process repeated twice after small aliquots of organic solvents had been added to redissolve the lipid. Further repetition of this process did not decrease the background. Attempts to dilute this background with unlabeled CCl_4 were negative, suggesting that the binding was not due to CCl_4 but perhaps some labeled contaminant. Labeled phosgene, a possible contaminant, has not been ruled out.

Purification of Cytochromes P_{450}, P_{448}, and b_5 from Rat Liver Microsomes

Purified cytochromes P_{450}, P_{448}, and b_5 are required for the investigation of two possible reactions resulting in CCl_4 activation, one in which these hemoproteins participate as pro-oxidants and the other involving electron capture from the reduced hemoproteins. These investigations require, as a minimum, that each is free of contamination from NADPH-cytochrome P_{450} reductase and other hemoproteins. Several methods are now available which yield cytochrome P_{450} preparations of apparent homogeneity. The procedure of Guengerich (1977) has been used for these investigations because of its relatively high yields of cytochrome P_{450} which is free of cytochrome b_5 and NADPH-cytochrome P_{450} reductase contamination. The procedure has another advantage in that

cytochrome P_{450} reductase and cytochrome b_5 can be separated and obtained as by-products of the preparation. A summary of a typical purification by this procedure is presented in Table 10. Some difficulties were encountered in reproducing the procedure. The peak fractions from the octylamine hydrophobic columns were of similar specific content to that published, 6.8 nmole P_{450}/mg protein; however, the peak fractions from the hydroxyapatite columns were not. Typical preparations yielded cytochrome P_{450} of lower specific content than that published for this step, from 7.5 to 10.0 nmole/mg instead of 13.3 nmole/mg. All conditions and procedures for carrying out this part of the purification procedures were carefully checked but cytochrome P_{450} preparation of equal purity to that published has not been obtained. Lever (1977) has recently reported that several non-ionic detergents containing polyether side chains were contaminated with peroxides. The detergent used in these purifications, Emulgen 913, is of similar chemical structure and has been found to contain low peroxide levels. Heme destruction due to the presence of these peroxides may increase the proportion of apoprotein in these purified preparations and therefore account for the relatively poor results obtained. The DEAE chromatography steps were omitted from this purification procedure because it did not greatly increase the specific content of the cytochrome P_{450} preparation and reduced significantly the final yield of the cytochrome. Electrophoresis of the purified fractions on polyacrylamide gels containing SDS was performed and the profiles are

TABLE 10.--Purification of Cytochrome P_{450} from Microsomes of PB-Pretreated Rats.

Fraction	Proteins (mg)	Total Content (nmoles)	Specific Content (nmoles/mg)	Yield (%)
Washed PB-microsomes	1,559	2,993	1.92	100
77,000 x g cholate solubilized supernatant	1,414	2,899	2.05	97
Octylamine column fractions, peak A	127	850	6.73	28
Octylamine column fractions, peak B	171	1,009	5.90	34
1st Hydroxyapatite column, 90 mM eluate	30.1	298	9.88	10
2nd Hydroxyapatite column, 90 mM eluate	16.3	148	9.08	5
Hydroxyapatite column, 150 mM eluate (pooled from columns 1 and 2, above)	4.3	30	6.91	1

shown in Figure 3. Gel #1 of the 90 mM fraction from PB-P_{450} was relatively free of contaminants but its specific content, 9.88 nmole/mg, indicated that it was only approximately 50% pure which supports the presence of apo-protein in this preparation.

Similar procedures were used to purify cytochrome P_{448} from liver microsomes of rats pretreated with 3-MC. The specific content of peak fractions from hydroxyapatite chromatography eluted with 90 mM and 150 mM K phosphate buffer was 7.62 and 5.39 nmole/mg, respectively. Electrophoresis profiles of these fractions (Figure 3, gels #3 and #4), show contamination with other proteins which accounts in part for the even lower specific content. Both the cytochrome P_{450} and P_{448} preparations were free of NADPH-cytochrome P_{450} reductase and cytochrome b_5. Only trace amounts of NADH-cytochrome b_5 reductase were found in the 150 mM cytochrome P_{450} and in the 90mM and 150 mM cytochrome P_{448} fractions.

Cytochrome b_5 was eluted from the octylamine columns according to the procedure of Imai (1976). Peak fractions were concentrated and treated as described in the Methods section of this chapter to remove the bulk of the detergents, sodium cholate, and deoxycholate. The final preparation had a specific content of 19.8 nmole cyt b_5/mg protein, which is approximately 30% pure. It did contain a major unidentified contaminant in the 50,000 to 60,000 dalton range (Figure 3, gel #5) which was determined spectrally not to be a cytochrome P_{450} hemoprotein.

Figure 3.--SDS-Polyacrylamide Gel Electrophoresis of Cytochromes P_{450}, P_{448}, and b_5 Fractions Purified from PB- and 3-MC-Microsomes.

Cytochromes P_{450} and b_5 were purified from PB-microsomes and cytochrome P_{448} from 3-MC-microsomes as described in Methods. Samples were applied to the gels as follows: (1) 20 μg cytochrome P_{450}, 90 mM fraction; (2) 20 μg cytochrome P_{450}, 150 mM fraction; (3) 20 μg cytochrome P_{448}, 90 mM fraction; (4) 10 μg cytochrome P_{448}, 150 mM fraction; and (5) 20 μg cytochrome b_5. The hemoprotein content of each fraction was 9.88, 6.91, 7.62, 5.39, and 19.98, respectively. Electrophoresis was conducted in the presence of 1% SDS and gels stained with Coomassie blue as described in Methods.

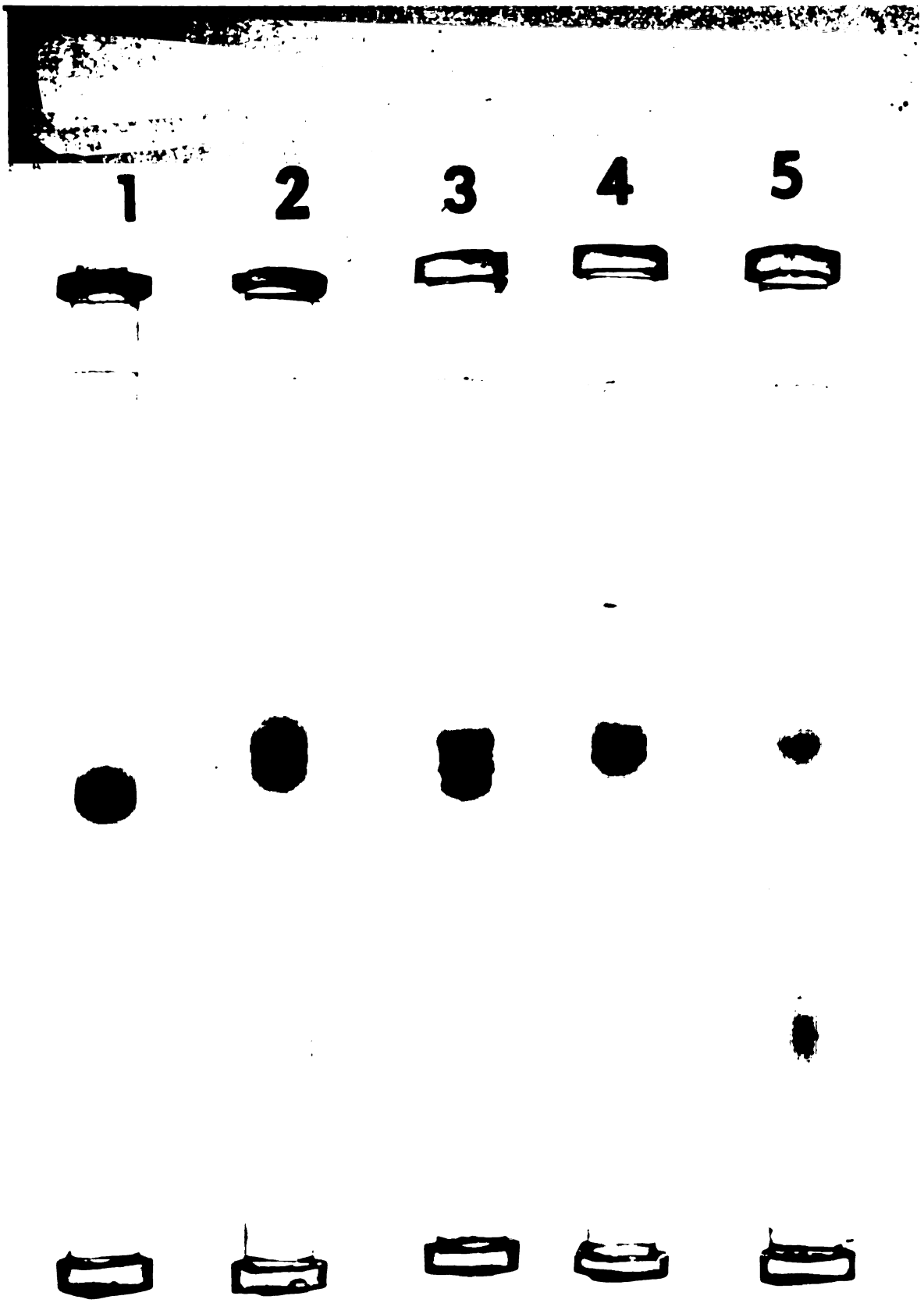


Fig. 3

The Role of Cellular Pro-Oxidants in CCl_4 Activation: Implications of the Function of This Mechanism in Vivo

The pro-oxidant, EDTA-Fe^{+2}, catalyzes the degradation of lipid hydroperoxides into free radicals capable of activating CCl_4. If this reaction is to be of any importance *in vivo*, cellular pro-oxidants including hemin, cytochrome P_{450} or other hemoproteins in their ferric form, and transition metals, should be equally effective as EDTA-Fe^{+2}. The results of an experiment which investigated the effect of CCl_4 on lipid peroxidation in the presence of cellular pro-oxidants are presented in Table 11. As shown, a CCl_4-dependent enhancement of lipid peroxidation occurs in liposomes containing protease-solubilized NADPH-cytochrome P_{450} reductase, ADP-Fe^{+3}, and either cytochromes P_{450}, P_{448}, b_5, methemoglobin, or hemin as pro-oxidants. The protease solubilized reductase cannot reduce cytochrome P_{450} or any of the other hemoproteins (Lu et al., 1969). It does, however, catalyze the initial formation of lipid hydroperoxides via the reduction of ADP-Fe^{+3} (Svingen et al., 1978).

The ability of these hemoproteins to activate CCl_4 in the presence of peroxidized lipid was investigated and the results are shown in Table 12 and Figure 4. The binding of $^{14}CCl_4$ with time in a system containing cytochrome P_{450} and partially peroxidized liposomes (0.0914 μmole LOOH/μmole lipid-PO_4) as shown in Figure 4. After a rapid initial increase, binding was linear for approximately 45 minutes. In a similar system (Table 12), several pro-oxidants

TABLE 11.--CCl_4-Stimulated Lipid Peroxidation in Liposomes in the Presence of Hemoproteins and Hemin.

Reactions contained 0.5 μmole lipid-PO_4/ml; 0.02 unit/ml NADPH-cytochrome P_{450} reductase (protease solubilized), ± 0.3 nmole heme/ml of either cytochromes P_{450}, P_{448}, and b_5, methemoglobin, or hemin, 1.66 mM ADP with 0.1 mM $FeCl_3$, 0.25 M NaCl, ± 1 μl CCl_4/ml, ± 0.3 mM NADPH in 0.1 M Tris-HCl, pH 7.4 at 37°C. Reactions were conducted as described in Methods for 15 min at 37°C.

Description	nmole MDA/15 min/ml		Stimulation
	(A) + NADPH	(B) + NADPH + CCl_4	(B/A)
Experiment 1			
Liposomes + ADP-Fe^{+3}	0.70	0.68	0.97
Liposomes + ADP-Fe^{+3} + 0.3 nmole/ml cytochrome b_5	0.84	0.90	1.07
Liposomes + ADP-Fe^{+3} + 0.3 nmole/ml hemin	1.08	1.42	1.31
Liposomes + ADP-Fe^{+3} + 0.3 nmole/ml methemoglobin	0.80	1.09	1.36
Experiment 2			
Liposomes + ADP-Fe^{+3}	0.57	0.89	1.56
Liposomes + ADP-Fe^{+3} + 0.3 nmole/ml cytochrome P_{450}	0.86	2.24	2.60
Liposomes + ADP-Fe^{+3} + 0.3 nmole/ml cytochrome P_{448}	2.12	2.41	1.14

TABLE 12.--Effect of Hemoproteins on $^{14}CCl_4$ Activation in Partially Peroxidized Liposomes.

Reactions contained 1.0 μmole partially peroxidized lipid-PO_4/ml (0.098 μmole LOOH/μmole lipid-PO_4), ± 0.5 or 2.5 nmole heme/ml hemoprotein or hemin, 0.05% sodium deoxycholate, 1 μl $^{14}CCl_4$/ml (0.25 m Ci/ml CCl_4) in 0.1 M Tris-HCl, pH 7.4 at 37°C. Reactions were conducted anaerobically as described in Methods for 30 min at 37°C.

Description	cpm/mg Lipid ± S.D.	Expt'l.-Cont.
Liposomal System		
No additions	2,002 ± 62	--
+ 0.5 nmole/ml cytochrome P_{450}	2,341 ± 88	339*
+ 2.5 nmole/ml cytochrome P_{450}	2,474 ± 189	472*
+ 2.5 nmole/ml cytochrome P_{448}	2,723 ± 878	721
+ 2.5 nmole/ml methemoglobin	2,403 ± 39	401*
+ 2.5 nmole/ml hemin	2,475 ± 178	473*

*Significantly different from control, $p < 0.05$.

Figure 4.--Activation of $^{14}CCl_4$ by Cytochrome P_{450} in Partially Peroxidized Liposomes.

Reactions contained 1.0 μmole partially peroxidized lipid-PO_4/ml (0.0913 μmole LOOH/μmole lipid-PO_4), 2.5 nmole/ml cytochrome P_{450}, 0.05% sodium deoxycholate, 0.75 l $^{14}CCl_4$/ml (0.25 m Ci/ml CCl_4) in 0.1 M Tris-HCl, pH 7.4 at 37°C. Reactions were performed in duplicate under anaerobic conditions as described in Methods for 2 to 45 min at 37°C.

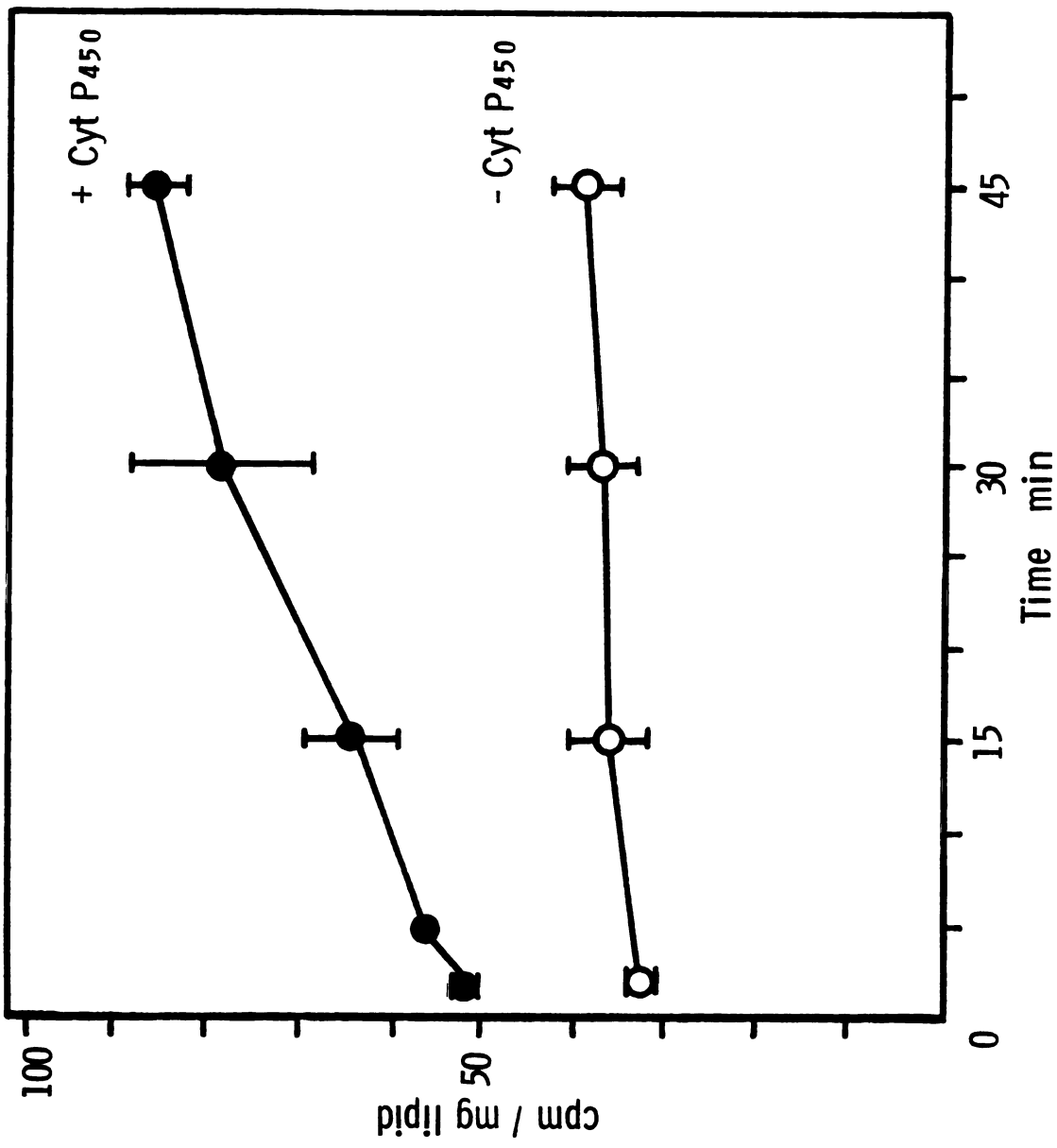


Fig. 4.

tested also increased binding; however, hemin was the more effective pro-oxidant.

The Effect of the Electron Acceptors ADP-Fe^{+3} and EDTA-Fe^{+3} on CCl_4 Activation by Rat Liver Microsomes

Activation of CCl_4 by the pro-oxidant reactions requires sources of lipid hydroperoxides and pro-oxidants. In microsomes lipid hydroperoxides can be generated by the NADPH-dependent reduction of ADP- or pyrophosphate-chelated iron (Pryor, 1976; Svingen et al., 1978). The oxidized microsomal hemoproteins, cytochromes $P_{450}(Fe^{+3})$ and b_5 (Fe^{+3}), have pro-oxidant activity and conditions which increase steady-state levels of this form therefore favor CCl_4 activation by pro-oxidant mechanisms. Other electron acceptors, $K_3Fe(CN)_6$ and cytochrome c, are believed to inhibit CCl_4 activation by channeling electrons away from cytochrome P_{450} (Glende and Recknagel, 1969). If the same were true for ADP-Fe^{+3} *in vivo*, the pro-oxidant mechanism would be favored. Since cytochrome P_{450} is concomitantly destroyed in these reactions, the net effect of this iron form would be inhibitory. It was therefore desirable to test the effect of the chelated iron on the rate of microsomal CCl_4 activation under anaerobic conditions. Figure 5 shows that the reaction was fairly linear in control rat liver microsomes for up to approximately 20 minutes. Fifteen-minute assays were used to determine the effects of ADP-Fe^{+3} and EDTA-Fe^{+3} on CCl_4 activation (Table 13). ADP-Fe^{+3} did not decrease CCl_4

Figure 5.--$^{14}CCl_4$ Activation by Rat Liver Microsomes.

Reactions contained 2.5 mg protein/ml (washed microsomes from unpretreated rats), 1 µl $^{14}CCl_4$/ml (0.25 m Ci/ml CCl_4), ± 0.3 mM NADPH in 0.1 M Tris-HCl, pH 7.4 at 37°C. Microsomes contained the following quantities of electron transport components: 0.65 nmole/mg cytochrome P_{450}; 0.41 nmole/mg cytochrome b_5; and 0.178 unit/mg NADPH-cytochrome P_{450} reductase. Reactions were conducted under anaerobic conditions as described in Methods from 5 to 60 min at 37°C.

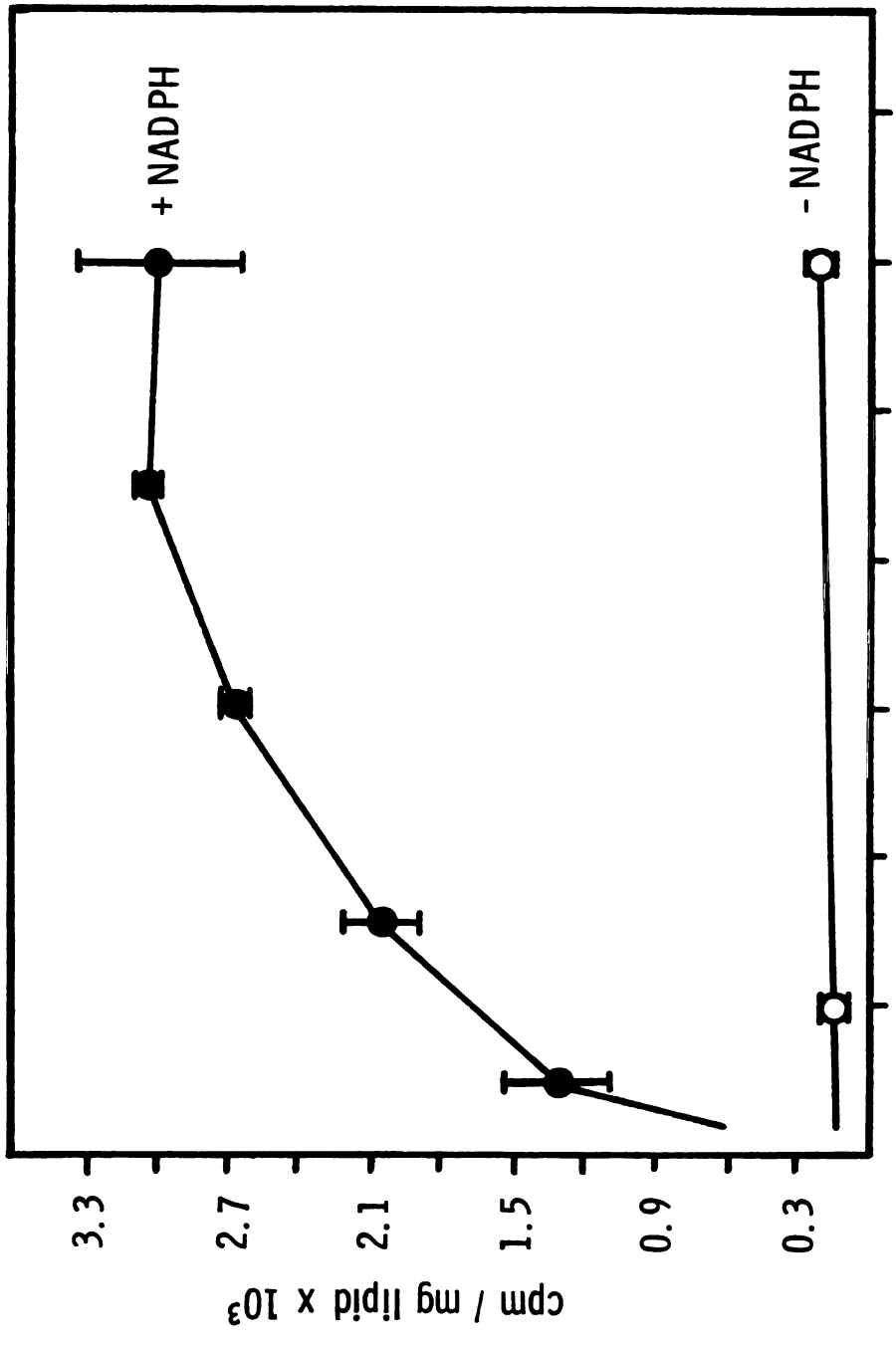

+ NADPH
- NADPH
cpm / mg lipid x 10³
3.3
2.7
2.1
1.5
0.9
0.3
0
10
20
30
40
50
60
70
Time min

Fig. 5.

TABLE 13.--Effect of ADP- and EDTA-Chelated Iron on $^{14}CCl_4$ Activation by Rat Liver Microsomes.

Reaction contained 2.5 mg protein/ml (washed microsomes from unpretreated rats), 1 µl $^{14}CCl_4$/ml (0.25 m Ci/ml CCl_4), ± 1.66 mM ADP with 0.1 mM $FeCl_3$, ± 0.5 mM EDTA with 0.5 mM $FeCl_3$, ± CO, ± 0.3 mM NADPH in 0.1 M Tris-HCl, pH 7.4 at 37°C. Reactions were conducted under anaerobic conditions as described in Methods for 15 minutes at 37°C.

Description		cpm/mg Lipid ± S.D.	Expt'l.-Cont.*
Washed microsomes	- NADPH	80 ± 9	
	+ NADPH	2,125 ± 285	2,045
Washed microsomes + ADP-Fe^{+3}	- NADPH	95 ± 8	
	+ NADPH	2,833 ± 61	2,738
Washed microsomes + EDTA-Fe^{+3}	- NADPH	72 ± 12	
	+ NADPH	613 ± 93	541
Washed microsomes + ADP-Fe^{+3} + EDTA-Fe^{+3}	- NADPH	87 ± 5	
	+ NADPH	582 ± 174	495
Washed microsomes + CO + NADPH		551 ± 51	471
Washed microsomes + CO + NADPH + ADP-Fe^{+3}		517 ± 9	422
Washed microsomes + CO + NADPH + EDTA-Fe^{+3}		315 ± 68	243
Washed microsomes + CO + ADP-Fe^{+3} + EDTA-Fe^{+3}		356 ± 112	269

*All significantly different from control, $p < 0.05$.

activation but instead gave an unexplainable increase (34%) in activation. Chelation of iron by ADP, a weak chelator, does not significantly alter the reduction potential of free iron; both Fe^{+3} and ADP-Fe^{+3} are reduced slowly by cytochrome P_{450} reductase. Iron chelated by EDTA, a strong chelator, on the other hand has an increased reduction potential and is therefore more easily reduced. Its rate of reduction was found to be ten-fold that of ADP-Fe^{+3} (Noguchi and Nakano, 1974). This would make EDTA-Fe^{+3} a better competitor for reducing equivalents from cytochrome P_{450} reductase and hence more inhibitory as observed in Table 13.

It is not likely that the increase in microsomal CCl_4 activation observed in the presence of ADP-Fe^{+3} is due to a pro-oxidant mechanism or to electron capture from ADP-Fe^{+2}. These possibilities would be reflected as an increased amount of CO-independent, and therefore cytochrome P_{450} (Fe^{+2})-independent, binding. The fact that no significant difference in labeling occurred in liposomes with low hydroperoxide content (Table 9), and a failure to detect a difference in the CO-insensitive labeling between control microsomes and microsomes and ADP-Fe^{+3}, rules out both possibilities.

Discussion

Slater and Sawyer (1971b, 1977) have proposed that NADPH-cytochrome P_{450} reductase is the site of CCl_4 activation within the microsomal mixed-function oxidase system. This hypothesis is based primarily on indirect evidence. The failure of several mixed-function oxidase inhibitors to decrease the CCl_4-dependent

microsomal lipid peroxidation was taken as proof of the reductase components' involvement. The interpretation of their data is complicated by the multiplicity of cytochrome P_{450} hemoproteins (Guengerich, 1977; Lu et al., 1976) and the fact that none of the inhibitors decreased mixed-function oxidase activity by more than 50 to 60% (Slater and Sawyer, 1971b). Differences in substrate specificities have been demonstrated for several forms which have been purified and it is not unlikely that they may also differ with respect to their reactivity with inhibitors (Gillette, 1971b; Mannering, 1971). For example, the cytochrome P_{450} hemoprotein which is most active in CCl_4 activation may not be inhibited sufficiently by the compounds used. The use of aminopyrine as an indicator of mixed-function oxidase activity assumes the P_{450} hemoprotein primarily involved in CCl_4 activation and that with specificity toward this compound are the same. This assumption may not be valid.

If the initiation of lipid peroxidation, via CCl_4 activation, is not the rate-limiting step involved in the stimulation of MDA production and the activation is not completely blocked at the cytochrome P_{450} site by mixed-function oxidase inhibitors, then the interpretation of the experimental results of Slater and Sawyer (1971b) as being due to CCl_4 activation by cytochrome P_{450} is questionable. The fact that the stimulatory effect of CCl_4 is not observed in the presence of high endogenous rates of lipid peroxidation (MDA formation) suggests that initiation is not rate limiting (Glende and Recknagel, 1969; Masuda and Murano, 1977). An

alternative interpretation of the results reported by Slater and Sawyer (1971b) would be that CCl_4 is activated in rat liver microsomes by reactions which require NADPH-cytochrome P_{450} reductase and microsomal components other than cytochrome P_{450}. This possibility will be further discussed in the following chapter.

In contrast to that hypothesis formulated by Slater and Sawyer (1971b), a direct activation of CCl_4 by NADPH-cytochrome P_{450} reductase has been ruled out by the investigations reported in this chapter. NADPH-cytochrome P_{450} reductase is converted to its fully reduced form in the presence of a 70-fold or greater excess of NADPH (Iyanagi et al., 1974; Williams and Kamin, 1962). The experimental conditions used to obtain the results summarized by Tables 2 and 3 favored full reduction of the enzyme. However, the lack of CCl_4 activation indicates that the proposed reaction involving electron capture from this form of the enzyme is unlikely.

NADPH-cytochrome P_{450} reductase in its half-reduced state contains the flavin semiquinone radical which has been postulated to be capable of CCl_4 activation by an atom transfer reaction (Slater, 1974). This form of the enzyme exists in the presence of NADPH and electron acceptors (Iyanagi et al., 1974); however, the lack of $^{14}CCl_4$ binding (Table 9) in the absence of lipid hydroperoxides does not support this hypothesis. The flavin semiquinone radical is resonance stabilized (Iyanagi et al., 1974; Walling, 1957) and may not be reactive enough to activate CCl_4 via an atom transfer reaction or to generate other radicals, e.g., by hydrogen abstraction from lipid, which can.

The more probable functions for NADPH cytochrome P_{450} reductase in CCl_4 activation are those which are indirect. On the one hand, it transfers reducing equivalents to cytochrome P_{450} which can activate CCl_4 directly. Alternatively, it can reduce iron chelated by ADP or pyrophosphate, which in the presence of oxygen initiates the formation of lipid hydroperoxides. Subsequently, these lipid hydroperoxides can be degraded by cellular pro-oxidants into lipid radicals capable of activating CCl_4. The results shown in Tables 10 and 11 support this view.

Cytochrome P_{450} and, to some extent, cytochrome b_5 are both destroyed during CCl_4 intoxication (Smuckler, 1976). Glende et al. (1977) have recently demonstrated a requirement for lipid peroxidation for cytochrome P_{450} destruction and concomitant loss of mixed-function oxidase activity. These findings suggest that a pro-oxidant mechanism of CCl_4 activation by the microsomal mixed-function oxidase system takes place in vivo. Since cytochrome P_{450} is the major microsomal hemoprotein and represents a significant portion of total microsomal protein, ranging from 4 to 20%, respectively, in untreated and PB-pretreated rats, this mechanism could be of some significance. The relative proportions of different cytochrome P_{450} hemoproteins may also be of importance. For example, a greater stimulation of lipid peroxidation is observed with cytochrome P_{450} than with cytochromes P_{448} or b_5 (Table 11). O'Brien and Rahimtula (1975) have also observed differences in the pro-oxidant activities for several hemoproteins in the presence of cumene hydroperoxide.

Though lipid peroxidation has been shown to occur normally in animals (Koster et al., 1977; Lindstrom and Anders, 1978), endogenous levels of hydroperoxides and pro-oxidants would probably not be high enough to account for the rapid initial rates of CCl_4 activation observed *in vivo* (Recknagel et al., 1977). Of the two indirect roles proposed for the function of NADPH-cytochrome P_{450} reductase in CCl_4 activation, that of greater importance is its reduction of cytochrome P_{450}. The results presented in Figures 4 and 5 allow for a comparison between rates of CCl_4 activation by reactions involving the pro-oxidant, $P_{450}(Fe^{+3})$, and enzymatically reduced, $P_{450}(Fe^{+2})$, form of the hemoprotein. The initial rate of activation by the reduced cytochrome P_{450} is much higher than that occurring by the pro-oxidant mechanism, 2.40 and approximately 0.06 nmoles $^{14}CCl_4$ bound/min/nmole P_{450}, respectively. *In vivo*, the presence of iron chelates such as ADP-Fe^{+3} may have a similar influence on activation via the reduced cytochrome P_{450} as that shown in Table 13; however, the magnitude of this effect has not been evaluated. Although of little importance during the initial stages of CCl_4 intoxication, activation via pro-oxidant-mediated reactions would become increasingly important as pathological damage increases. For example, the increase in lipid hydroperoxides (Recknagel et al., 1977) and in microsomal iron content (Thiers et al., 1960) and a decreased rate of cytochrome P_{450} reduction in damaged microsomal membranes (Archakov and Karuzina, 1973) all favor CCl_4 activation via pro-oxidant-mediated reactions.

The results of the investigations presented in this chapter have excluded a direct role for cytochrome P_{450} reductase in CCl_4 activation. Two possible indirect functions in CCl_4 activation have been presented and their relative importance considered. Cytochrome P_{450} reductase's involvement in pro-oxidant-mediated reactions which activate CCl_4 have been discussed in some detail. Its involvement in the reduced cytochrome P_{450}-mediated and other possible reactions which activate CCl_4 will be discussed in the following chapters.

CHAPTER III

THE ROLE OF THE CYTOCHROME P_{450} COMPONENT OF RAT LIVER MICROSOMES IN CARBON TETRACHLORIDE ACTIVATION

Abstract

Washed microsomes containing different cytochrome P_{450} levels were used in investigations to determine its role in CCl_4 activation. $CoCl_2$ pretreatment of rats reduced the cytochrome P_{450} level but did not alter the cytochrome P_{450} reductase content of their liver microsomes. The cytochrome P_{450} hemoproteins of $CoCl_2$-microsomes were qualitatively similar to those of unpretreated microsomes. Both the rates of endogenous and CCl_4-stimulated lipid peroxidation as well as the extent of lipid peroxidation correlated with microsomal cytochrome P_{450} contents. A reconstituted microsomal mixed-function oxidase system containing liposomes, cytochrome P_{450}, and cytochrome P_{450} reductase was used to investigate the role of cytochrome P_{450} in $^{14}CCl_4$ activation. An NADPH-dependent binding of $^{14}CCl_4$ to liposomes was observed. Carbon monoxide and an antibody to the major PB-inducible cytochrome P_{450} inhibited binding, thus confirming the role of this hemoprotein in $^{14}CCl_4$ activation. Binding to liposomes also occurred when NADPH-cytochrome P_{450} reductase and NADPH were replaced by dithionite as a source of reducing equivalents for cytochrome P_{450}. Other hemoproteins and hemin worked in this system as well.

An investigation of alternate pathways of CCl_4 activation by microsomes was undertaken. A cytochrome b_5-dependent $^{14}CCl_4$ binding to microsomal lipid was observed in microsomes in the presence of CO with NADH as cofactor. An additional CO-insensitive NADPH-dependent binding of $^{14}CCl_4$ to microsomal lipid was observed. This activity was greatly decreased in washed microsomes. Both alternate pathways could activate CCl_4 only at rates of less than 10% of the cytochrome P_{450}-dependent rate and are believed to be of little consequence in vivo.

Introduction

CCl_4 is activated by the liver microsomal mixed-function oxidase system; however, there has been some uncertainty concerning the role of its two components, cytochrome P_{450} and NADPH-cytochrome P_{450} reductase, in this activation. It has been suggested that either one or the other of these components may function as the site of CCl_4 activation (Recknagel et al., 1977; Slater, 1972; Slater and Sawyer, 1971b). For example, Recknagel et al. (1977) had formulated an hypothesis which suggested that the reduced cytochrome P_{450} activated CCl_4 by an electron capture reaction. The evidence in support of the reductase as being the site of activation has been discussed in Chapter II; that in support of cytochrome P_{450} is presented below.

Many mixed-function oxidase substrates bind the ferric form of cytochrome P_{450} to give the characteristic Type I or Type II binding spectra (Mannering, 1971). The addition of CCl_4 to

microsomes results in a Type I binding spectrum. Uehleke et al. (1973) and Wolf et al. (1977) have obtained spectral evidence for the interaction of CCl_4 with the ferrous form of cytochrome P_{450}. Characteristic difference spectra with absorbance maxima ranging from 454 to 470 nm resulted when CCl_4 and other halomethanes were added to reduced microsomes. Treatment of rats with compounds which alter liver microsomal cytochrome P_{450} levels similarly affects CCl_4 toxicity. Pretreatment of rats with inducers of cytochrome P_{450}, PB and DDT, enhance CCl_4 metabolism and toxicity (Recknagel and Glende, 1973). On the other hand, treatments which decrease microsomal cytochrome P_{450} levels ($CoCl_2$, allylisopropyl acetamide, and sublethal doses of CCl_4) also decrease toxicity (Glende, 1972; Sipes et al., 1977; Suriyachian and Thithapandha, 1977). Newborn rats have low cytochrome P_{450} levels but close to their adult complement of NADPH-cytochrome P_{450} reductase. They are resistant to the toxic effects of CCl_4 but become more susceptible as their cytochrome content of the liver approaches the adult levels (Recknagel et al., 1977). Metyrapone and CO inhibit microsomal mixed-function oxidase activity through interaction with cytochrome P_{450}. Both were found to inhibit CCl_4 activation in vitro when measured either by the binding of $^{14}CCl_4$ to microsomal proteins and lipids or by $CHCl_3$ formation under anaerobic conditions (Villarruel et al., 1975; Uehleke et al., 1973).

Chemical evidence which supports a role for a hemoprotein in the activation of CCl_4 via an electron capture reaction has

been presented. Wade and Castro (1973, 1974) have demonstrated that reduced heme compounds and some hemoproteins are oxidized by halogenated alkanes. The products of these reactions were those expected to occur from the dimerization of two dehalogenated alkyl radicals. Hemoproteins with the axial ligands of heme iron accessible were capable of catalyzing these reactions but those in which the protein conformation restricted ligand accessibility were not (Wade and Castro, 1974). These findings not only provide a possible chemical explanation for the activation of CCl_4 by cytochrome P_{450} but leave open the possibility of activation by the hemoprotein, cytochrome b_5, as well. Cytochrome b_5 can be reduced *in vivo* by either NADPH-cytochrome P_{450} reductase or NADH-cytochrome b_5 reductase (Archakov et al., 1975) and subsequently transfer reducing equivalents to cytochrome P_{450} (Fujita and Peisac, 1977; Mannering, 1974). Though the participation of cytochrome b_5 in CCl_4 activation by this route is possible, the direct activation by the reduced hemoprotein cannot be excluded.

Included in this chapter are the results of a series of investigations undertaken to confirm the role of cytochrome P_{450} as the primary site of CCl_4 activation within the microsomal mixed-function oxidase system. Three approaches were used in the course of these investigations. The first was to determine if a relationship between CCl_4 activation and microsomal cytochrome P_{450} content existed *in vitro*. Pretreatment of rats with $CoCl_2$ lowers cytochrome P_{450} content without affecting the

NADPH-cytochrome P_{450} reductase component and was therefore utilized in these investigations. The second approach was to utilize a reconstituted mixed-function oxidase system to investigate the function of cytochrome P_{450} in CCl_4 activation. Since the results in Chapter II showed that NADPH-cytochrome P_{450} reductase does not activate CCl_4, activation by the reconstituted system would confirm a role for cytochrome P_{450}. Reactions which include other microsomal components may also function to activate CCl_4. If these reactions are significant, they may provide an alternative interpretation of the data of Slater and Sawyer (1971b) which suggested that NADPH-cytochrome P_{450} reductase was the site of CCl_4 activation. The third approach therefore involves the investigation of CCl_4 activation in microsomes in which cytochrome P_{450} mediated reactions have been inhibited.

Methods and Materials

Material Sources

Male Sprague-Dawley rats weighing between 200 and 225 g were obtained from Spartan Research Animals, Inc., Haslett, Michigan.

Benzphetamine was a gift from Dr. P. W. O'Connel of the Upjohn Co., Kalamazoo, Michigan. Cumene hydroperoxide (CHP) was from K&K Laboratories, Plainview, New York. Catalase (2x recrystalyzed) and NADH were from the Sigma Chemical Co., St. Louis, Missouri. 2,4-Pentanedione was from the Aldrich Chemical Co.,

Milwaukee, Wisconsin. Freund's adjuvant (complete) was from Difco Laboratories, Detroit, Michigan.

The sources of other agents used have been listed in Chapter II.

Drug Pretreatments of Rats

Rats were pretreated with PB as described in Chapter II. Other pretreatments were with either $CoCl_2$ (80 mg/kg in saline) by s.c. injection 48 and 24 hr prior to sacrifice or with CCl_4 (5 ml/kg of a 20% solution of CCl_4 in corn oil) by a single i.p. injection 24 hr prior to sacrifice. The controls were similarly injected with equal volumes of saline or corn oil, respectively. Microsomes isolated from rats pretreated with either the vehicle or the above chemicals are referred to as either control (C)-, PB-, $CoCl_2$-, or CCl_4-microsomes.

Isolation of Rat Liver Microsomes

Microsomes were isolated from PB- and CCl_4-pretreated rats and their respective controls according to the procedure of Pederson (1973). They were washed by 25-fold dilution with 1mM Tris-HCl (pH 7.5 at 25°C) and recentrifugation, and stored as described in Chapter II in the absence of BHT. Microsomes from untreated and $CoCl_2$-pretreated rats were isolated as above, washed with argon-saturated solution of 1.15% kCl and 0.2% nicotinamide, and stored as described. Microsomes were used within 7 days after isolation.

Reconstitution of Mixed-Function Oxidase Activity from Purified Components

The reconstituted mixed-function oxidase system contained the following: 0.1 mg di-1,2-GPC/ml; 0.200 units/ml NADPH-cytochrome P_{450} reductase; 0.4 nmole/ml cytochrome P_{450} (90 mM fraction from PB-microsomes); 100 µg/ml sodium deoxycholate; 0.1 mM EDTA; 0.01 M $MgCl_2$; 15 units/ml catalase (passed through a Sephadex G25 column to remove preservatives) 0.1 mM NADPH; and ± 1.0 mM benzphetamine in 0.1 M Tris-HCl, pH 7.4 at 37°C. The lipid, NADPH-cytochrome P_{450} reductase, and cytochrome P_{450} components were first added to the quartz cuvettes (either 1 or 0.5 ml capacity), thoroughly mixed and allowed to incubate for 10 minutes at 2-4°C. The remaining components were then added and the mixture allowed to stand for an additional 10 minutes (15 minutes if either the preimmune or immune IgG against cytochrome P_{450} was also included). Benzphetamine demethylation assays were then conducted at 37°C as follows: cuvettes were preincubated for 4 minutes at 37°C; NADPH was added and basal rate of NADPH oxidation followed at 340 nm; and after the basal rate was established, benzphetamine (20 mM in H_2O) was added to give a final concentration of the monitoring of 1 mM and NADPH oxidation continued. The extinction coefficient of $\varepsilon = 6.22\ mM^{-1}cm^{-1}$ (Horecker and Kornberg, 1948) was used to calculate the rate of benzphetamine demethylation.

A similar system was used for $^{14}CCl_4$ activation with the modifications listed below. The reactions were prepared and run in

a 10-ml Erlenmyer flask. Catalase was omitted and liposomes, 1.0 μmole lipid-PO_4/ml, were included along with buffer and other components. The flasks were sealed under argon and made anaerobic as described in Chapter II. $^{14}CCl_4$ was subsequently added, via Hamilton syringe, at the concentrations indicated. After a 3-minute preincubation, the reactions were initiated with the addition of NADPH. The remaining procedures have been described in Chapter II.

When required, CO was added to the sealed anaerobic system as follows. The flasks were made anaerobic, a vacuum established within, and CO gas added through the rubber seal by a hypodermic needle attached to tank regulator via tygon tubing. After the contents of the flasks were under slightly positive CO pressure, a second needle was inserted and gas allowed to flow through the open-system for 30 seconds. Both needles were then removed leaving the flasks under slight CO-positive pressure.

Enzyme Assays and Analytical Procedures

Microsomal mixed-function oxidase activity was determined by two methods. The benzphetamine-dependent oxidation of NADPH by microsomes was followed as described above or by the measurement of formaldehyde formation according to the method of Nash (1953). The procedure described by Pederson (1973) was used with the substitution of benzphetamine (2.5 mM) for aminopyrine.

Lipid peroxidation was measured by determining MDA formation as outlined in Chapter II. Reactions contained 1 mg/ml

microsomal protein, ± 1 µl CCl_4/ml, and ± 0.3 mM NADPH or 0.19 mM CuOOH in chelex-treated 0.1 M Tris-HCl, pH 7.4 at 37°C. Iron was omitted from these reactions. Reactions were initiated with either NADPH or CHP (in acetone) and run for designated periods of time at 37°C.

Dithionite Reduction of Microsomes, Hemoproteins and Hemin

When required, stock sodium dithionite solutions (2 mM) were prepared by adding crystals to argon-saturated borate buffer, 25 mM, pH 9.0. Reactions were initiated by adding dithionite, in 8- to 16-fold excess, via Hamilton syringe.

Preparation of Antibody to Trypsin-Solubilized Cytochrome P_{450}

The preparation of the antigen (trypsin-solubilized cytochrome P_{450}), immunization of the rabbits, and preparation of IgG were as described previously (Welton, 1974).

Results

The Relationship Between the Cytochrome P_{450} Content of Rat Liver Microsomes and CCl_4-Dependent Lipid Peroxidation

Support for the hypothesis which assigns cytochrome P_{450} as the site of CCl_4 activation stems primarily from the fact that changes in its microsomal content also alter CCl_4 activation and toxicity (Recknagel and Glende, 1973). Several compounds, such as PB, DDT, and CCl_4, are known to alter microsomal cytochrome P_{450} content. However, their effectiveness as tools to be used in

investigating CCl_4 activation by cytochrome P_{450} is limited by the fact that they also affect other microsomal constituents (Glende et al., 1976; Parke, 1975). Cobaltous chloride pretreatment decreases cellular levels of cytochrome P_{450} with little effect on other microsomal components and was used for these investigations (Guzelian and Bissell, 1976; Suarez and Bhonsle, 1976).

There are two possibilities for the participation of cytochrome P_{450} in CCl_4 activation and toxicity. One involves the direct activation of CCl_4 by the reduced hemoprotein and the other is due to the pro-oxidant activity of the oxidized hemoprotein. As a pro-oxidant cytochrome P_{450} propagates lipid peroxidation which results in additional CCl_4 activation. Both reactions involving CCl_4 activation function to increase lipid peroxidation and perhaps toxicity. The stimulation of lipid peroxidation in microsomes containing different cytochrome P_{450} levels was investigated (Figure 6) to determine its role as the site for CCl_4 activation. A relationship was demonstrated between microsomal cytochrome P_{450} contents and both the endogenous and CCl_4-dependent rates of lipid peroxidation. There is approximately 3.5-fold more cytochrome P_{450} (2.9-fold more total heme) in control than there is in $CoCl_2$-microsomes (Table 14). Similarly, the initial rates of endogenous and CCl_4-dependent lipid peroxidation are 3.4- and 2.9-fold greater, respectively, for control- than for $CoCl_2$-microsomes (inset of Figure 6). The stimulation of lipid peroxidation by CCl_4 was similar for both control- and

Figure 6.--Stimulation of Lipid Peroxidation by CCl_4 in Liver Microsomes from Unpretreated and $CoCl_2$ Pretreated Rats.

Reactions contained 1 mg protein/ml (washed microsomes), 1 µl CCl_4/ml, ± 0.3 mM NADPH in 0.05 M Tris-HCl, pH 7.4 at 37°C. Reactions were conducted at 37°C and MDA determined as described in Methods.

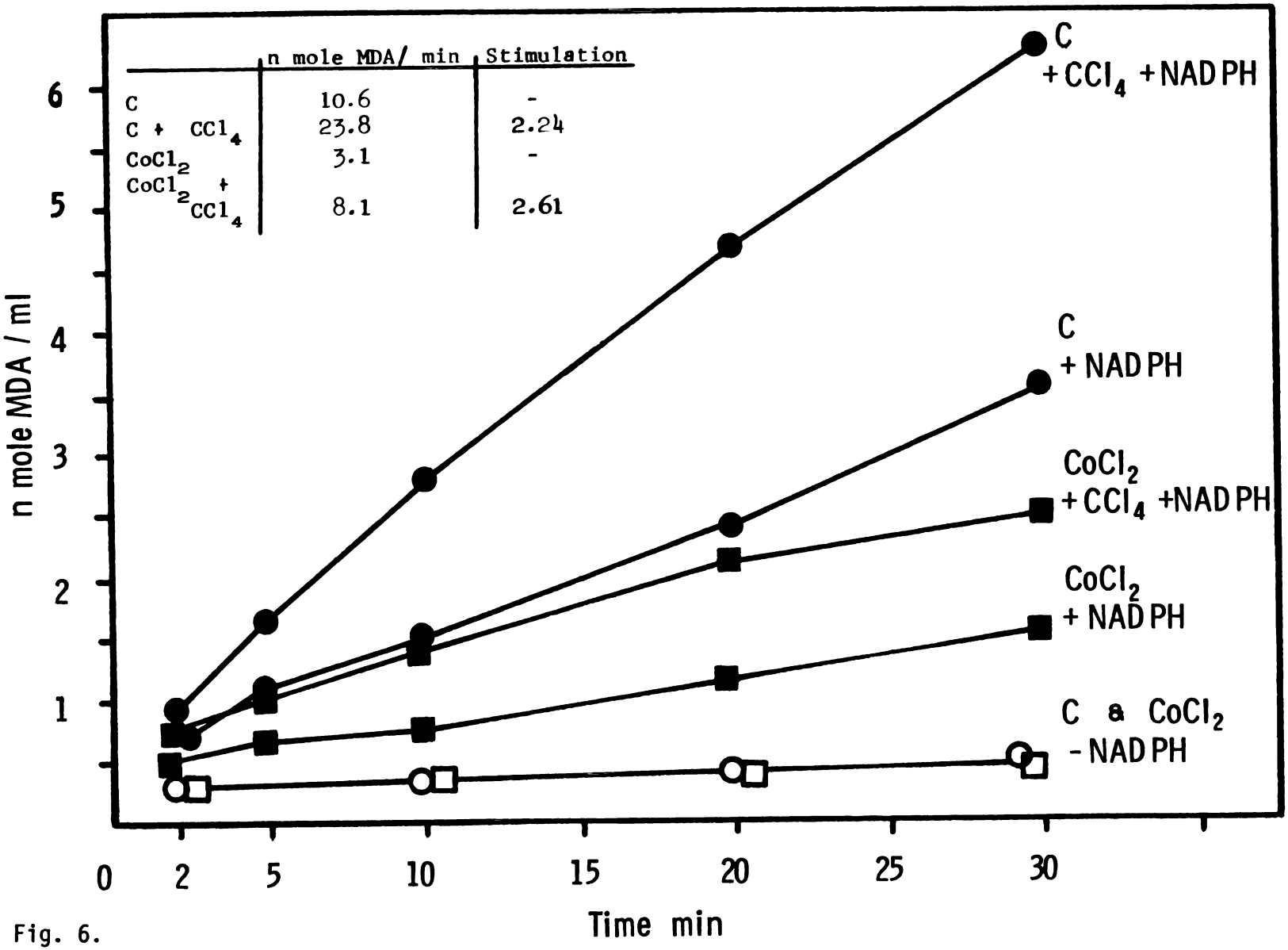

n mole MDA/ min
Stimulation
C
10.6
-
C + CCl4
23.8
2.24
CoCl2
3.1
-
CoCl2 + CCl4
8.1
2.61
C + CCl4 + NADPH
C + NADPH
CoCl2 + CCl4 + NADPH
CoCl2 + NADPH
C & CoCl2 - NADPH
n mole MDA / ml
Time min
0
2
5
10
15
20
25
30
1
2
3
4
5
6

Fig. 6.

TABLE 14.--The Effect of $CoCl_2$ Pretreatment on Rat Liver Microsomal Enzymes.

Rats were injected, s.c., with either $CoCl_2$ (80 mg/kg in saline) or saline at 48 and 24 hours prior to sacrifice. After sacrifice the livers were perfused, homogenized, and microsomes isolated by differential centrifugation in the presence of 1.15% KCl and 0.2% nicotinamide. Microsomes were washed once by suspension and recentrifugation in the KCl-nicotinamide.

Pretreatment	Cytochrome P_{450} (nmole/mg)	Cytochrome b_5 (nmole/mg)	Reductase* (units/mg)
Microsomes			
Control (saline)	0.879	0.537	0.263
$CoCl_2$	0.249	0.219	0.216

*Units of NADPH-cytochrome P_{450} reductase equal μmoles cytochrome c reduced/min.

$CoCl_2$-microsomes, 2.25- and 2.62-fold stimulation, respectively. These findings demonstrate a relationship between microsomal cytochrome P_{450} content and CCl_4 activation and suggest that there are no qualitative differences between the cytochrome P_{450} hemoproteins of control and $CoCl_2$ microsomes. Such qualitative differences may arise, for example, if $CoCl_2$ causes a preferential decrease in one or more cytochrome P_{450} hemoproteins in rat liver microsomes.

Cytochrome P_{450} is also a pro-oxidant which can function to further promote lipid peroxidation (O'Brien, 1975). Therefore a relationship between the pro-oxidant activity and cytochrome P_{450} content of microsomes should also exist. This relationship can be investigated in the absence of initiation reactions by the addition

of excess cumene hydroperoxide, CHP. Figure 7 shows that a correlation exists between both the rate and extent of lipid peroxidation and microsomal cytochrome P_{450} content. The curves reached a plateau at approximately 8.2 and 2.7 nmole/MDA/10 min/ml, respectively, for control- and $CoCl_2$-microsomes, a 3-fold difference. This probably reflects the fact that cytochrome P_{450} and, to some degree, cytochrome b_5 are destroyed during lipid peroxidation (Smuckler, 1976) thereby limiting the autocatalytic phase of the reaction.

Activation of CCl_4 by a Reconstituted Mixed-Function Oxidase System Containing Purified Components from PB-Microsomes

Support for the hypothesis designating cytochrome P_{450} as the site of CCl_4 activation within the microsomal mixed-function oxidase system has all been indirect (Recknagel et al., 1977). Since previous investigations have ruled out NADPH-cytochrome P_{450} reductase as the activation site (Chapter II), activation by a reconstituted mixed-function oxidase system would confirm this role for cytochrome P_{450}. A reconstituted system composed of mixed-function oxidase components purified from PB-microsomes was used in these investigations. Table 15 shows the metabolism of benzphetamine, a substrate preferentially metabolized by the major PB-inducible cytochrome P_{450} hemoprotein, by the reconstituted system (Guengerich, 1977). The rate of metabolism obtained in this system (turnover number of 44.7 nmoles NADPH oxidized/min/

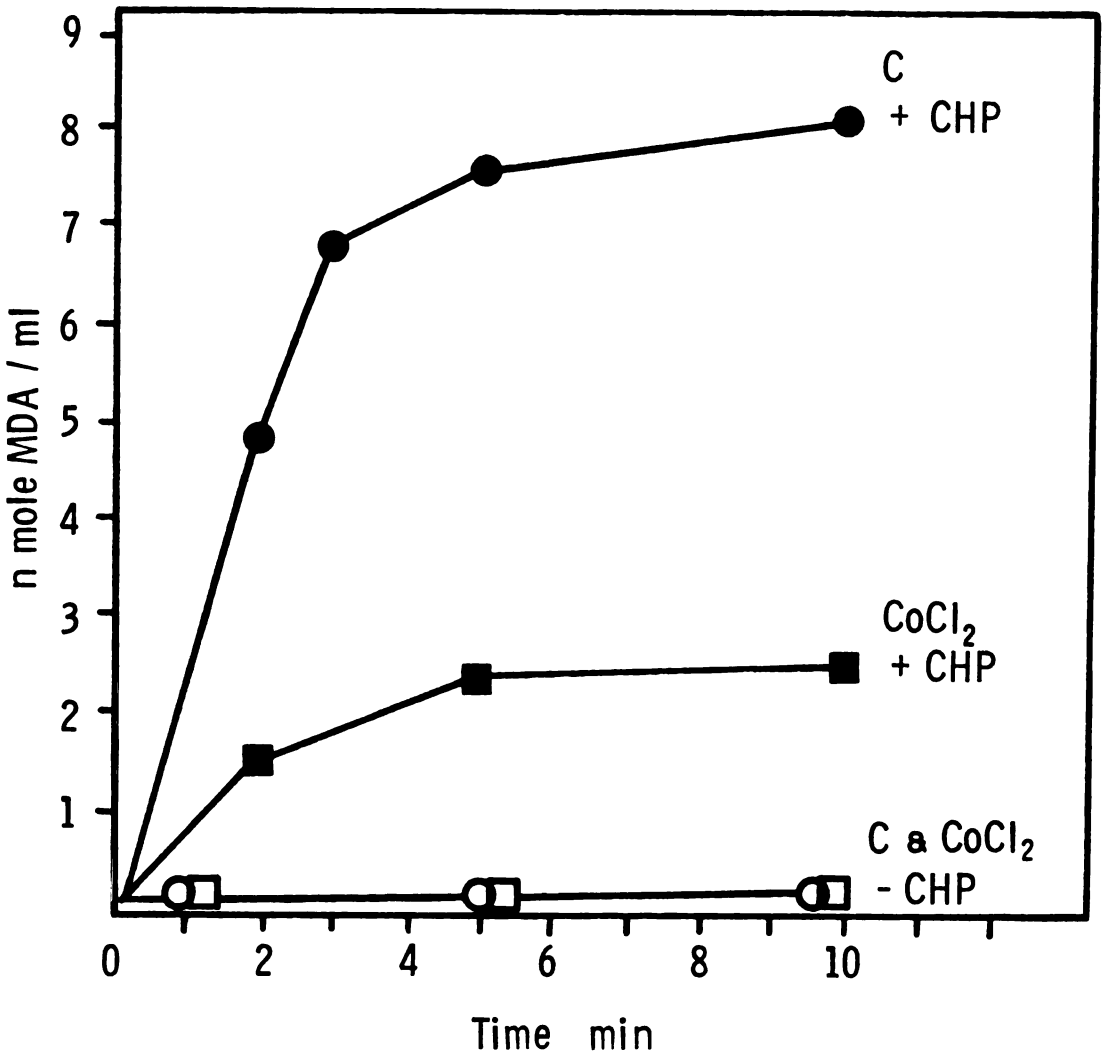


Figure 7.--CHP-Dependent Lipid Peroxidation in Liver Microsomes from Untreated and $CoCl_2$ Pretreated Rats.

Reactions contained 0.63 mg protein/ml (washed microsomes), ± 0.19 mM CHP in 0.05 M Tris-HCl, pH 7.4 at 37°C. Reactions were conducted at 37°C and MDA determined as described in Methods.

TABLE 15.--Reconstitution of Mixed-Function Oxidase Activity: Inhibition of Activity With Antibody Against Cytochrome P_{450}.

Reactions contained 0.1 mg/ml di-1,2-GPC, 0.20 units/ml NADPH-cytochrome P_{450} reductase, 0.4 nmole/ml cytochrome P_{450} (9.88 nmole/mg), 15 units/ml catalase, 0.1 mM EDTA, 0.010 M $MgCl_2$, 100 µg/ml sodium deoxycholate, 1.0 mM benzphetamine, and 0.1 mM NADPH in 0.1M Tris-HCl, pH 7.4 at 37°C. Assay was conducted as described in Methods.

Description	Benzphetamine* Demethylation (nmole/min)	% Cont.
Complete system	17.9	100.0
+ Immune IgG 2:1 mg IgG/nmole Cyt-P_{450}	15.9	88.8
+ Immune IgG 5:1 mg IgG/nmole Cyt-P_{450}	12.5	69.8
+ Immune IgG 10:1 mg IgG/nmole Cyt-P_{450}	7.9	44.1
+ Immune IgG 20:1 mg IgG/nmole Cyt-P_{450}	3.4	19.0
+ Preimmune IgG 10:1 mg IgG/nmole Cyt-P_{450}	22.4	125.1
+ Preimmune IgG 20:1 mg IgG/nmole Cyt-P_{450}	22.5	125.7

*nmoles benzphetamine-dependent oxidation of NADPH/min/ml.

mole cyt P_{450}) was comparable to that previously reported (Kamataki et al., 1976; van der Hoeven and Coon, 1974). An antibody specific for the PB-inducible cytochrome P_{450} (Welton et al., 1975) was tested for its effect on benzphetamine demethylation. At relatively high protein levels (20 mg IgG/nmole cyt P_{450}), 80% inhibition was achieved with this antibody.

That the reconstituted mixed-function oxidase system is capable of CCl_4 activation is demonstrated by Figure 8. Liposomes prepared from extracted microsomal lipids were added to a similar reconstituted system as that previously used and the anaerobic binding of $^{14}CCl_4$ to this added lipid used to estimate activation. The rate of $^{14}CCl_4$ binding, 0.37 nmole $^{14}CCl_4$/min/nmole cyt P_{450}, was less than that previously determined in control microsomes (Figure 5), approximately 2.4 nmole $^{14}CCl_4$/min/nmole cyt P_{450}. The reason for this discrepancy is uncertain; however, it may be due to the relatively excessive amounts of lipid (1.0 μmole lipid PO_4/ml assay) required for measurable binding. Lipid has previously been reported to be inhibitory if added in too great an excess (Lu and Levin, 1974).

The results of two separate investigations which determined the effect of inhibitors of mixed-function oxidase activity on CCl_4 activation are presented in Table 16. The first, performed earlier in my work on this problem, utilized relatively inhomogenous cytochrome P_{450} (4.0 nmole cyt P_{450}/mg) prepared by a procedure (Lu and Levin, 1972) which differed from that described in the Methods section. Experiment 2 utilized the more purified cytochrome P_{450} and NADPH-cytochrome P_{450} reductase preparation described in Methods. Carbon monixide and immune IgG were both inhibitory (Table 16). The immune IgG used in Experiment 1 was of higher titer than that in Experiment 2 and thus required less protein for substantial inhibition of benzphetamine demethylation. At the levels used, immune IgG

Figure 8.--$^{14}CCl_4$ Activation by a Reconstituted Microsomal Mixed-Function Oxidase System.

Reaction contained 0.1 mg/ml di-1,2,-GPC, 0.20 unit/ml NADPH-cytochrome P_{450} reductase, 0.4 nmole/ml cytochrome P_{450} (9.88 nmole/mg), 0.1 mM EDTA, 10 mM $MgCl_2$, 0.01% sodium deoxycholate, 1.0 μmole lipid-PO_4/ml, 1 μl $^{14}CCl_4$/ml (0.25 m Ci/ml CCl_4) ± 0.3 mM NADPH in 0.1 M Tris-HCl, pH 7.4 at 37°C. Reactions were conducted under anaerobic conditions as described in Methods at 37°C.

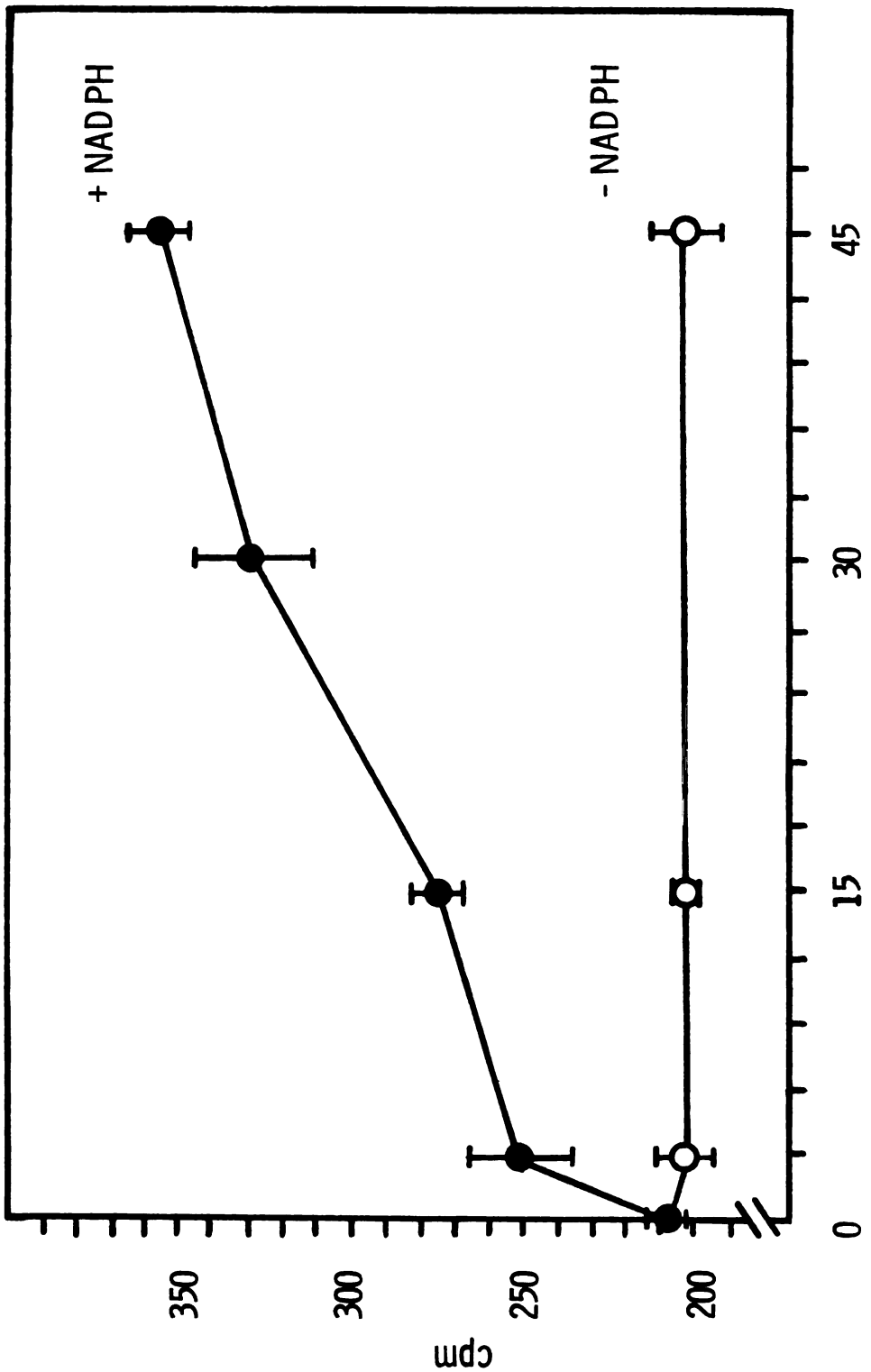


Fig. 8

TABLE 16.--Effect of Inhibitors of Drug Metabolism on $^{14}CCl_4$ Activation by a Reconstituted Mixed-Function Oxidase System.

Experiment 1. Reactions contained 50 µg/ml di-1,2-GPC, 0.150 units/ml NADPH-cytochrome P_{450} reductase, 0.4 nmole/ml cytochrome P_{450} (4 nmole cyt P_{450}/mg), 1 µl $^{14}CCl_4$/ml (0.25 m Ci/ml CCl_4), and other components described in Methods.

Experiment 2. Reactions contained di-1,2-GPC, NADPH-cytochrome P_{450} reductase, cytochrome P450 (9.88 nmole cyt P_{450}/mg), 1 µl $^{14}CCl_4$/ml (0.25 m Ci/ml CCl_4) and other components as described in Methods. Experiments 1 and 2 were run for 60 and 30 minutes, respectively, under anaerobic conditions according to Methods. IgG content expressed as ratio of mg IgG:nmole cyt P_{450}.

Description	cpm	± S.D.	Expt'l.-Cont.
Experiment 1			
Complete - NADPH	876	± 112	--
Complete + NADPH	1,269	± 131	393*
Complete + NADPH + CO	937	± 71	61
Complete + NADPH + Preimmune IgG (2:1)	1,012	± 61	136
Complete + NADPH + Immune IgG (2:1)	846	± 140	< 0
Experiment 2			
Complete - NADPH	2,509	± 55	--
Complete + NADPH	2,756	± 134	247*
Complete + NADPH + CO	2,641	± 154	132
Complete + NADPH + Preimmune IgG (10:1)	2,663	± 61	154
Complete + NADPH + Immune IgG (10:1)	2,491	± 164	< 0

*Significantly different from control, $p < 0.05$.

inhibited 93 and 56% of benzphetamine demethylation by the reconstituted systems used in Experiments 1 and 2, respectively. Both preimmune and immune IgG inhibited $^{14}CCl_4$ binding, however, the latter was consistently the more effective inhibitor. That the preimmune IgG was also inhibitory is not unexpected since it can compete with lipid for $^{14}CCl_4$ binding. Inhibition by CO was variable but in both experiments decreased $^{14}CCl_4$ activation to levels not significantly different from the non-enzymatic labeling.

Activation of CCl_4 by Dithionite-Reduced Cytochrome P_{450} and Other Hemoproteins

Under normal conditions cytochrome P_{450} is reduced by the NADPH-cytochrome P_{450} reductase-mediated transfer of reducing equivalents from NADPH. Guengerich et al. (1975) have demonstrated that dithionite will serve as a source of reducing equivalents in the metabolism of some drugs, though less efficiently than when cytochrome P_{450} was enzymatically reduced. It is also possible that dithionite may serve as a source of reducing equivalents for CCl_4 activation, thereby further demonstrating that cytochrome P_{450} is responsible for this activity. Although some investigators have reported little or no CCl_4 activation by dithionite-reduced microsomes, the activation of alkyl halides by other chemically reduced hemoproteins encouraged further pursuit of these experiments (Wade and Castro, 1973, 1974).

Table 17 shows an experiment where excess dithionite was added to an anaerobic suspension of washed rat liver microsomes and

TABLE 17.--Activation of $^{14}CCl_4$ by NADPH-Cytochrome P_{450} Reductase- and Dithionite-Reduced Cytochrome P_{450} in Microsomes.

Reactions contained 2.5 mg/ml washed microsomal protein from unpretreated rats, 1 µl $^{14}CCl_4$/ml (0.25 mCi/ml CCl_4), and either ± 0.25 mM NADPH or + 50 µM dithionite in 0.1 M Tris-HCl, pH 7.4 at 35°C. Reactions were conducted under anaerobic conditions as described in Methods for 20 min at 37°C.

Description	cpm/mg Lipid ± S.D.	Expt'l.-Cont.
Control Microsomes		
Control - NADPH	40 ± 8	--
Control + NADPH	2,343 ± 82	2,303*
Control + 50 µM dithionite	295 ± 12	255*

*Significantly different from control, $p < 0.01$.

the extent of activation determined. Some labeling was achieved although it was only 10% of that for NADPH-reduced microsomes over the same 20-minute time period. Table 18 contains the results of experiments in which CCl_4 activation by several hemoproteins and hemin was investigated. Dithionite gave some background labeling; however, each of the hemoproteins tested resulted in labeling above this background. The labeling obtained in the presence of methemoglobin and hemin suggests that a specific substrate binding site is not required but interaction with the heme iron is required for CCl_4 activation.

TABLE 18.--Activation of $^{14}CCl_4$ by Dithionite-Reduced Hemoproteins and Hemin in Liposomes.

Reactions contained 1.0 µmole lipid-PO_4/ml, 0.01% sodium deoxycholate, 1 µl $^{14}CCl_4$/ml (0.25 m Ci/ml CCl_4), 3 or 6 nmole heme/ml from either hemoproteins or hemin, ± 50 µM dithionite in 0.1 M Tris-HCl, pH 7.4 at 37°C. Reactions were conducted under anaerobic conditions as described in Methods for 30 min at 37°C.

Description	cpm/mg Lipid ± S.D.	Expt'l.-Cont.
Liposomes	1,185 ± 75	--
Liposomes + 50 µM dithionite	1,830 ± 153	--
Liposomes + 50 µM dithionite + 3 nmole/ml Cyt P_{450}	2,850 ± 247	1,020*
Liposomes + 50 µM dithionite + 6 nmole/ml Cyt P_{450}	3,589 ± 296	1,759*
Liposomes + 50 µM dithionite + 6 nmole/ml Cyt P_{448}	1,874 ± 488	44
Liposomes + 50 µM dithionite + 6 nmole/ml methemoglobin	2,542 ± 791	712
Liposomes + 50 µM dithionite + 6 nmole/ml hemin	4,058 ± 1,116	2,228

*Significantly different from control (liposomes + dithionite), $p < 0.05$.

The Role of Cytochrome b_5 in the NADH-Dependent Activation of CCl_4 in Microsomes

The NADH-dependent microsomal electron transport chain has generally been thought not to play a major role in CCl_4 activation (Slater, 1971a). Glende and Recknagel (1969) have demonstrated that CCl_4-dependent stimulation of microsomal lipid peroxidation occurs with NADH as a cofactor but at a rate 25% of that observed with NADPH. This stimulation may be attributed to an interaction between the NADH- and NADPH-dependent microsomal electron transport chains. NADH-cytochrome b_5 reductase could transfer reducing equivalents from NADH to cytochrome b_5 which subsequently reduces cytochrome P_{450} (Mannering, 1974). Alternatively, reduced cytochrome b_5 may be capable of directly activating CCl_4. This may be tested experimentally by determining the NADH-dependent and CO-insensitive binding of $^{14}CCl_4$ to microsomal lipid. Carbon monoxide will not react with cytochrome b_5 but binds strongly to reduced cytochrome P_{450}. It would also be of interest to determine if NADH reacts synergistically with NADPH in CCl_4 metabolism as it does with other mixed-function oxidase substrates which give Type I binding spectra (Cohen and Estabrook, 1971; Mannering, 1974).

The experiments reported in Table 19 examined the effect of either NADPH, NADH, or both, on CCl_4 activation by washed untreated microsomes and the inhibition of this activation by CO. Synergism was not observed in the presence of both cofactors. The unexpected increase in activation due to NADH in the presence

TABLE 19.--NADH- and NADPH-Dependent Activation of $^{14}CCl_4$ in Microsomes.

Reaction contained 2.5 mg/ml washed microsomal protein from unpretreated rats, 1 µl $^{14}CCl_4$/ml (0.25 m Ci/ml CCl_4), ± CO, and either ± 0.25 mM NADPH, NADH, or both in 0.1 M Tris-HCl, pH 7.4 at 37°C. Reactions were conducted under anaerobic conditions as described in Methods for 20 min at 37°C.

Description	cpm/mg Lipid ± S.D.	Expt'l.-Cont.
Control Microsomes		
- NADPH	40 ± 8	--
+ NADPH	2,343 ± 82	2,303*
+ NADPH + CO	750 ± 42	710*
+ NADH	276 ± 38	236*
+ NADH + CO	420 ± 78	380*
+ NADH + NADPH	2,411 ± 303	2,371*
+ NADH + NADPH + CO	641 ± 9	601*

*Significantly different from control, $p < 0.05$.

of CO probably results from direct activation via cytochrome b_5. Under these assay conditions CO would form a stable complex with reduced cytochrome P_{450} thereby preventing its oxidation of cytochrome b_5. The levels of reduced cytochrome b_5 should increase favoring an increased activation by this hemoprotein.

The Effect of Washing on Microsomal CCl_4 Activation

The possibility of cellular constituents other than those normally associated with the liver microsomal mixed-function oxidase system function in CCl_4 activation has not been ruled out.

As shown in Table 13, it is possible that cellular constituents such as ADP- or pyrophosphate-chelated iron function *in vivo* to increase the activation of CCl_4 by the microsomal mixed-function oxidase system. In any investigations which seek to correlate microsomal cytochrome P_{450} levels with CCl_4 activation, one must take into account the effects of these constituents on the interpretation of data. This would be especially true if their presence was variable due to animal pretreatments or differences in microsomal isolation procedures. Table 20 contains the results of two experiments which determined the effect of washing on CCl_4 activation by PB- and CCl_4-microsomes and their respective controls. A buffer of low ionic strength which had previously been treated with a chelating resin to remove trace iron contamination was used for washing. A similar treatment has been shown to be very effective in reducing trace iron contamination of microsomes (Poyer and McCay, 1971). In both experiments washing decreased the amount of CO-insensitive, and presumed cytochrome P_{450}-independent, CCl_4 activation. After washing a better correlation of CCl_4 activation with cytochrome P_{450} levels resulted; this is especially true for Experiment 1. The reasons for these differences have not been determined; however, it was demonstrated that washing either does not alter the level of mixed-function oxidase components and drug metabolism (not shown) or gives a slight increase when compared to unwashed on a protein basis (Table 21).

TABLE 20.--The Effect of Washing on the CO-Insensitive $^{14}CCl_4$ Activation by Liver Microsomes from PB- and CCl_4-Pretreated Rats.

Microsomes were isolated from PB- and CCl_4-pretreated rats and their respective controls and washed with 1 mM Tris-HCl, pH 7.5 at 25°C, as described in Methods. Reactions contained 2.5 mg/ml microsomal protein, 1 µl $^{14}CCl_4$/ml (0.25 mCi/ml CCl_4), ± 0.3 mM NADPH, and ± CO in 0.1 M Tris-HCl, pH 7.4 at 37°C. Reactions were conducted anaerobically as described in Methods and run for 15 min at 37°C.

Pretreatment	$^{14}CCl_4$ Bound* (cpm/mg Lipid)				% Control
	(A)	(-) CO	(B)	(+) CO	(B/A)
Experiment 1					
Control (saline) unwashed		2,992		160	5.3
Control (saline) washed		1,420		7	0.5
PB unwashed		2,767		551	19.9
PB washed		2,444		19	0.8
Experiment 2					
Control (corn oil) unwashed		1,946		717	36.8
Control (corn oil) washed		2,413		574	23.8
CCl_4 unwashed		1,159		197	17.0
CCl_4 washed		1,251		138	11.0

*Difference between cpm bound in presence and absence of NADPH.

TABLE 21.--The Effect of Washing on Microsomal Enzymes from PB- and CCl_4-Pretreated Rats.

Microsomes isolated from PB- and CCl_4-pretreated rats and their respective controls were either used in these experiments without further treatment or after washing with 1 mM Tris-HCl, pH 7.5 at 25°C, as described in Methods. Enzyme assays and analytical techniques used to characterize these microsomes have been described in Methods.

Pretreatment	Cyt. P_{450} (nmole/mg)	Cyt. b_5 (nmole/mg)	Cyt. P_{450}* Reductase (unit/mg)
Experiment 1			
Control (saline) unwashed	1.020	0.517	0.378
Control (saline) washed	0.988	0.543	0.434
PB unwashed	1.560	0.810	0.553
PB washed	2.292	0.610	0.631
Experiment 2			
Control (corn oil) unwashed	0.507	0.309	0.168
Control (corn oil) washed	0.626	0.365	0.210
CCl_4 unwashed	0.246	0.253	0.126
CCl_4 washed	0.288	0.340	0.200

*1 unit = μmole cytochrome c reduced/min.

Discussion

The involvement of the cytochrome P_{450} component of the liver microsomal mixed-function oxidase system in CCl_4 activation has previously been suggested by Recknagel and Glende (1973). That this hemoprotein is the primary site of CCl_4 activation has been confirmed by the results of several experiments presented in this chapter. The rates of CCl_4-dependent lipid peroxidation (Figure 6) and the rate and extent of CHP-dependent lipid peroxidation (Figure 7) were related to the microsomal cytochrome P_{450} content (Table 14). These results are in agreement with the *in vivo* studies where chemical pretreatments were used to alter microsomal cytochrome P_{450} content which in turn altered CCl_4 toxicity (Glende and Recknagel, 1973; Lindstrom and Anders, 1978; Plaa and Witschi, 1976).

Cytochrome P_{450} has two functions in the CCl_4-dependent peroxidation of microsomal lipids. The reduced hemoprotein activates CCl_4 to $\cdot CCl_3$ which subsequently initiates lipid hydroperoxide formation. The oxidized hemoprotein functions as a pro-oxidant catalyzing the degradation of lipid hydroperoxides and subsequent propagation of more radical reactions. Since cytochrome P_{450} represents a substantial portion of liver microsomal protein, its relative distribution within an animal would be expected to have a significant bearing on CCl_4 toxicity. This interpretation, of course, holds only if there are no qualitative differences between the cytochrome P_{450} hemoproteins in a given animal or tissue. It

is possible for cytochrome P_{450} hemoproteins to differ in their interactions with CCl_4, in their peroxidase activity and in their pro-oxidant activity toward endogenous lipid hydroperoxides (Table 11). The aspect of CCl_4 activation by catalytically different cytochrome P_{450} hemoproteins will be discussed in greater depth in the following chapter.

There has been some lack of agreement between several investigators regarding the component responsible for CCl_4 activation within the mixed-function oxidase system. A combination of the experimental results presented in Chapter II with those presented in Table 16 and Figure 8 demonstrates the role of cytochrome P_{450} in CCl_4 activation. CCl_4 is activated only by the complete reconstituted mixed-function oxidase containing cytochrome P_{450}, NADPH-cytochrome P_{450} reductase, and liposomes. Activation is inhibited (Table 16) by CO and an antibody with specificity toward the major PB-induced cytochrome P_{450} hemoprotein (Welton et al., 1975). That cytochrome P_{450} is the site of CCl_4 activation is further demonstrated by the results of the experiments presented in Table 18. Dithionite will reduce cytochrome P_{450} and, as shown, can substitute for the enzymatic reduction of the hemoprotein in CCl_4 activation reactions.

Since CCl_4 gives a Type I binding spectrum in the presence of the oxidized cytochrome P_{450}, it was of interest to determine if this type of interaction was necessary for activation. That interaction with the Type I binding site may not be necessary has

been suggested by the effects of Type I substrates on CCl_4 activation. For example, the mixed-function oxidase inhibitor SKF-525A, a Type I substrate, has been found to either have no effect (Slater and Sawyer, 1971b; Masuda and Murano, 1977) or to slightly enhance CCl_4 activation (Uehleke et al., 1973). Stimulation of CCl_4 activation is probably related to the fact that this compound has been found to enhance the rate of cytochrome P_{450} reduction in microsomes (Suriyachan and Thithapanda, 1977). The fact that both methemoglobin and hemin will catalyze CCl_4 binding to liposomes (Table 18) suggests that interaction with the heme moiety of cytochrome P_{450} is required for activation. Carbon monoxide binds to the reduced heme iron; that it inhibits CCl_4 activation (Table 16) confirms an interaction between CCl_4 and the heme moiety of cytochrome P_{450}.

The chemical mechanisms which may be involved in the activation of CCl_4 by reduced cytochrome P_{450} are those initially proposed by Wade and Castro (1973, 1974) for the oxidation of alkyl halides by ferrous-porphyrin compounds and hemoproteins. They suggested the following sequence of reactions:

$$\begin{vmatrix}\\ Fe^{+2}\\ |\end{vmatrix} + RX \rightleftharpoons \begin{vmatrix}\\ Fe^{+2}(XR)\\ |\end{vmatrix} \xrightarrow{slow} \begin{vmatrix}\\ [Fe\text{---}X\text{---}R]^{+2}\\ |\end{vmatrix} \longrightarrow \begin{vmatrix}\\ Fe^{+3}X^-\\ |\end{vmatrix} + R\cdot$$

$$\begin{vmatrix}\\ Fe^{+3}X^-\\ |\end{vmatrix} + solvent \longrightarrow \begin{vmatrix}\\ Fe^{+3}(solvent)\\ |\end{vmatrix} + X^-$$

$$2R\cdot \longrightarrow R_2$$

By monitoring either the rates of hemoprotein oxidation or of dimer formation, they were able to demonstrate the dehalogenation of several compounds. Dehalogenation was catalyzed by all hemoproteins tested in which the axial positions of the heme iron were accessible to the substrates. That similar reactions are involved in the cytochrome P_{450} catalyzed metabolism of CCl_4 was suggested by Wolf et al. (1977). They detected CO formation from various halomethanes upon anaerobic incubation with NADPH- or dithionite-reduced microsomes. The reactions proposed to account for CO formation were as follows:

(1) $$CX_4 + Fe^{+2} \longrightarrow [Fe^{+3} \cdot CX_3] + X^-$$

(2) $$[Fe^{+3} \cdot CX_3] + e^- \longrightarrow [Fe^{+2} \cdot CX_3] \rightleftharpoons [Fe^{+3} : CX_3^-]$$

(3) $$[Fe^{+3} : CX_3^-] + e^- \longrightarrow [Fe^{+2} : CX_3^-] \longrightarrow [Fe^{+2} : CX_2] + X^-$$

(4) $$[Fe^{+2} : CX_2] + H_2O \longrightarrow Fe^{+2} + CO + 2\ HX$$

The dissociation of $\cdot CX_3$ from cytochrome P_{450}, reaction 1, would account for the increases in lipid peroxidation and for part of the binding to microsomal lipid and protein observed during CCl_4 intoxication. The formation of the carbene intermediate has been supported by Mansuy et al. (1977). They were able to demonstrate the formation of a stable carbene complex with the metalloporphyrin 5,10,15,20-tetraphenylporphinato iron (II).

The formation of a $\cdot CCl_3$ intermediate is also of interest in that its reaction with oxygen would provide a means by which both the toxic alkylating agent phosgene, $COCl_2$, and its hydrolysis product CO_2 may be formed (Rebbert and Ausloss, 1976; Slater, 1972). Although the detection of phosgene has not been reported, the expiration of $^{14}CO_2$ by animals administered $^{14}CCl_4$ has been demonstrated (Seawright and McLean, 1967). Dichlorocarbenes are also capable of binding to olefins forming cyclopropane derivatives (Kleveland et al., 1977). This in part may account for some of the observed binding to microsomal unsaturated lipids. This is not likely, however, to account for the observed toxicity in that not all halomethanes capable of carbene formation, for example, $CFCl_3$, are toxic (Cox et al., 1972; Wolf et al., 1977).

From this series of reactions, three possible reactive intermediates result from the cytochrome P_{450}-mediated reduction of CCl_4, $\cdot CCl_3$, $COCl_2$, and $:CCl_2$. The relative proportions of each produced would be related to CCl_4 toxicity. For example, toxicity can result from the binding of $\cdot CCl_3$ or $COCl_2$ to cellular components or from the ability of $\cdot CCl_3$ to initiate lipid peroxidation. Wolf et al. (1977) demonstrated that CO formation was at a rate approximately one-third that observed for NADPH oxidation. Whether the remaining intermediate products primarily consist of $\cdot CCl_3$ or $:CCl_2$ (and $COCl_2$ under aerobic conditions) has not been determined. Another observation of interest was the fact that microsomes from BP-pretreated rats produced CO at a faster rate than did those from

PB-pretreated rats. Whether these differences in cytochrome P_{450} hemoproteins can also account for differences in hepatoxicity will be discussed in Chapter IV.

Although cytochrome P_{450} has a major role in CCl_4 activation, other microsomal components may also participate. The lack of inhibition of CCl_4-stimulated lipid peroxidation by mixed-function oxidase inhibitors (Masuda and Murano, 1977; Slater and Sawyer, 1971b) suggest this possibility. Cytochrome b_5, for example, may be capable of CCl_4 activation. The increase in NADH-dependent CCl_4 activation in microsomes in the presence of CO (Table 19) supports this possibility. Activation by the reduced cytochrome b_5 would, however, be expected to be of little consequence in mediating CCl_4 toxicity when compared to the cytochrome P_{450}-dependent activation rates. In addition to activation by cytochrome b_5 another, CO-insensitive, component was found to be involved in CCl_4 activation. This component was removed by washing and may be or functions similarly to ADP-Fe^{+3} (Table 13). The identity of this component and its mechanism of action are not known.

CHAPTER IV

THE ACTIVATION OF CARBON TETRACHLORIDE BY CATALYTICALLY DIFFERENT CYTOCHROME P_{450} HEMOPROTEINS

Abstract

Liver microsomes from untreated rats or those pretreated with PB and 3-MC were utilized to determine if differences in CCl_4 activation and toxicity were related to the substrate specificites of their cytochrome P_{450} hemoproteins. Activation was determined by measuring both the CCl_4-dependent lipid peroxidation and the covalent binding of $^{14}CCl_4$ to microsomal lipid. The turnover numbers for CCl_4 activation by microsomes from untreated and pretreated rats were different. The relative magnitudes of these numbers for PB- and 3-MC-microsomes depended on the method used to detect CCl_4 activation. These differences are believed due to the relative amounts of $\cdot CCl_3$ and $:CCl_2$ produced by each microsomal cytochrome P_{450} hemoprotein population. For example, the more $\cdot CCl_3$ produced as an intermediate, the greater the turnover number from the assays which detect lipid peroxidation. Binding of $^{14}CCl_4$ to microsomal lipid is a better indicator of the rate of CCl_4 conversion to both $\cdot CCl_3$ and $:CCl_2$. The former initiates lipid peroxidation as well as binds to lipid whereas the latter is only capable of binding.

The turnover numbers for covalent binding of $^{14}CCl_4$ to microsomal lipid were more dependent on the NADPH-cytochrome P_{450} reductase to cytochrome P_{450} ratios than on qualitative differences in the cytochrome P_{450} hemoproteins. This was confirmed by the fact that reconstituted mixed-function oxidase systems containing catalytically different cytochrome P_{450} hemoproteins and the same amount of NADPH-cytochrome P_{450} reductase gave similar rates of $^{14}CCl_4$ binding.

It is concluded that several factors other than the differences in substrate specificity of the cytochrome P_{450} hemoproteins account for CCl_4 activation and toxicity. Included among these are the following: the microsomal content of cytochrome P_{450}; the rate of cytochrome P_{450} reduction; the relative amount of $\cdot CCl_3$ formed as an intermediate during CCl_4 metabolism *in vivo*; and cellular protective mechanisms. It is believed that either all or a combination of these factors are crucial in determining the susceptibility of various tissues and animal species to CCl_4 toxicity.

Introduction

The hypothesis which assigns cytochrome P_{450} as the site of CCl_4 activation within the liver microsomal mixed-function oxidase system (Recknagel and Glende, 1973; Recknagel et al., 1977) has been confirmed by the experimental results of Chapter III. A relationship between CCl_4 activation and relative amounts of microsomal cytochrome P_{450} has been observed by others (Recknagel and

Glende, 1973; Carlson, 1975), and the data presented in Chapter III, Figures 6 and 7, agrees with this observation. Mixed-function oxidase systems that activate CCl_4 have been demonstrated in a variety of tissues within the rat. However, the extent of activation as measured by $^{14}CCl_4$ binding to microsomal lipids does not correlate with cytochrome P_{450} levels in these tissues (Villarruel et al., 1977). This was true even though CCl_4 levels in these tissues were of similar magnitude. Diaz Gomez et al. (1975) examined the livers of several species for their susceptibility to CCl_4 toxicity. They were not able to correlate either $^{14}CCl_4$ binding, CCl_4-dependent increases in diene conjugation, or CCl_4-hepatotoxicity with cytochrome P_{450} levels. Species differences in the spectral binding constants for CCl_4 with cytochrome P_{450} were also unrelated to CCl_4 activation and hepatotoxicity. There are several plausible interpretations of these findings; one is that the multiple forms of cytochrome P_{450} differ in their ability to activate CCl_4. Another possibility would be due to differences in defense mechanisms which protect against CCl_4 hepatoxicity, for example, the cellular antioxidant content.

Several chemical compounds will induce cytochrome P_{450} hemoproteins with different substrate specificies. Pretreatment of animals with these compounds has been demonstrated to alter CCl_4 hepatotoxicity. For example, PB, PCN, and 3-MC induce cytochrome P_{450} hemoproteins in rats which are catalytically different from each other as well as from those in untreated rats.

PB enhances hepatoxicity whereas PCN and 3-MC, an inducer of cytochrome P_{448}, afford some protection (Castro et al., 1973; Recknagel and Glende, 1973; Suarez et al., 1975; Tuchweber et al., 1974). It is of interest that BP, which also induces cytochrome P_{448}, enhances rather than protects against CCl_4 hepatoxicity (Pitchumoni et al., 1972). The interpretation of these observations is complicated by the fact that they also alter several microsomal components, some of which may affect CCl_4 metabolism. For example, both PB and PCN cause a marked proliferation in the smooth endoplasmic reticulum and induce both of the mixed-function oxidase components and a broad spectrum of mixed-function oxidase activities (Parke, 1975; Tuchweber et al., 1974). Treatment with 3-MC or BP does not result in this proliferation and the spectrum of enzymatic activities induced is more limited. Both also induce the cytochrome P_{448} hemoprotein but only the latter enhances the cytochrome P_{450} reductase component (Parke, 1975). Metabolites of BP and 3-MC are antioxidants and if present in significant amounts would reduce peroxidative damage and perhaps toxicity (Pederson, 1973; Suarez et al., 1975).

The investigations presented in this chapter address two questions concerning CCl_4 activation: (1) Are the differences in CCl_4 toxicity observed in various tissues or animal species due to the substrate specificities of their cytochrome P_{450} hemoproteins? and (2) Are there other factors which influence CCl_4 toxicity in various tissues? The ability of microsomes from rats pretreated

with various compounds and of reconstituted systems containing cytochrome P_{450} purified from these microsomes to activate CCl_4 has been used in these investigations to answer these questions. The CCl_4-dependent lipid peroxidation and the binding of $^{14}CCl_4$ to liposomes have been used as an estimate of CCl_4 activation.

Materials and Methods

Material Sources

3,4-Benzpyrene was obtained from the Aldrich Chemical Co., Milwaukee, Wisconsin.

The sources of other reagents have been listed in Chapters II and III.

Drug Pretreatment of Rats

3-MC pretreatments have been described in Chapter II. PB (80 mg/kg in saline) was given to rats by daily injections, i.p., for 5 days prior to sacrifice. The microsomes isolated from rats injected with the saline or PEG vehicle or with PB or 3-MC are referred to as control (saline)-, control (PEG-), PB- and 3-MC-microsomes, respectively.

Isolation of Rat Liver Microsomes

Rats were starved for 16 hours prior to sacrifice and their liver microsomes isolated and washed as described in Chapter II but with the exceptions listed below. Livers were perfused with and homogenized in argon-saturated solution containing 1.15% KCl and 10 mM EDTA, pH 7.5. Microsomal pellets were washed once with

argon-saturated 1.15% KCl and stored without BHT as previously described, Chapter II. Microsomes were used within two days of preparation.

Enzyme Assays and Analytical Techniques

NADPH-cytochrome P_{450} reductase (Chapter II), benzphetamine demethylase (Chapter III), benzpyrene hydroxylase (Welton et al., 1975), and cytochromes P_{450} and b_5 (Omura and Sato, 1964) were assayed by previously described techniques. Benzphetamine and BP metabolism were determined at 37°C. All other assays were carried out at room temperature. MDA was determined as described in Chapter II. $^{14}CCl_4$ activation was determined by covalent binding to microsomal lipids as described in Chapter II with the modification that 2 mg/ml microsomal protein was used and the assays run anaerobically for 5 minutes. Activation of $^{14}CCl_4$ in liposomes was as described in Chapter III.

Results

Activation of CCl_4 by Microsomes from Untreated and PB- and 3-MC-Pretreated Rats

The failure of CCl_4 activation and toxicity to correlate with the cytochrome P_{450} levels found in various tissues and animal species may be related to qualitative differences in these hemoproteins. To test this hypothesis, rats were pretreated with compounds which alter both liver microsomal cytochrome P_{450} levels and substrate specificity and the activation of CCl_4 by these

microsomes determined. Two methods were used to estimate CCl_4 activation; one involved the stimulation of NADPH-dependent lipid peroxidation by CCl_4 and the other measured binding of $^{14}CCl_4$ to microsomal lipids. The effect of these pretreatments on microsomal enzymes and CCl_4-stimulated lipid peroxidation are shown in Table 22 and Figure 9. Endogenous rates of NADPH-dependent lipid peroxidation have been greatly reduced by the microsomal isolation and washing procedures described in the Methods section. This allows for better quantitation of the lipid peroxidation stemming from CCl_4 activation. PB pretreatment increased whereas 3-MC pretreatment of rats decreased the CCl_4-dependent lipid peroxidation when

TABLE 22.--The Effect of PB- and 3-MC Pretreatments of Rats on Microsomal Enzyme Content.

The pretreatment of rats, microsomal isolation and washing procedures, and enzymatic analysis were as described in Methods.

Pretreatment	Cyt. P_{450} (nmole/mg)	Cyt. b_5 (nmole/mg)	NADPH-Cyt. P_{450} Reductase (units/mg)*
Microsomes			
Control (saline)	0.756	0.385	0.295
PB	2.973	0.477	0.439
Control (PEG)	0.667	0.415	0.272
3-MC	1.157	0.476	0.264

*1 unit = μmole cytochrome c reduced/minute.

Figure 9.--Stimulation of Lipid Peroxidation by CCl_4 in Liver Microsomes from Untreated, PB-, and 3-MC-Pretreated Rats.

Reactions contained 1 mg protein/ml (washed microsomes) ± 1 µl CCl_4/ml, ± 0.3 mM NADPH in 0.1 M Tris-HCl, pH 7.4 at 37°C. Reactions were conducted at 37°C and MDA determined as described in Methods.

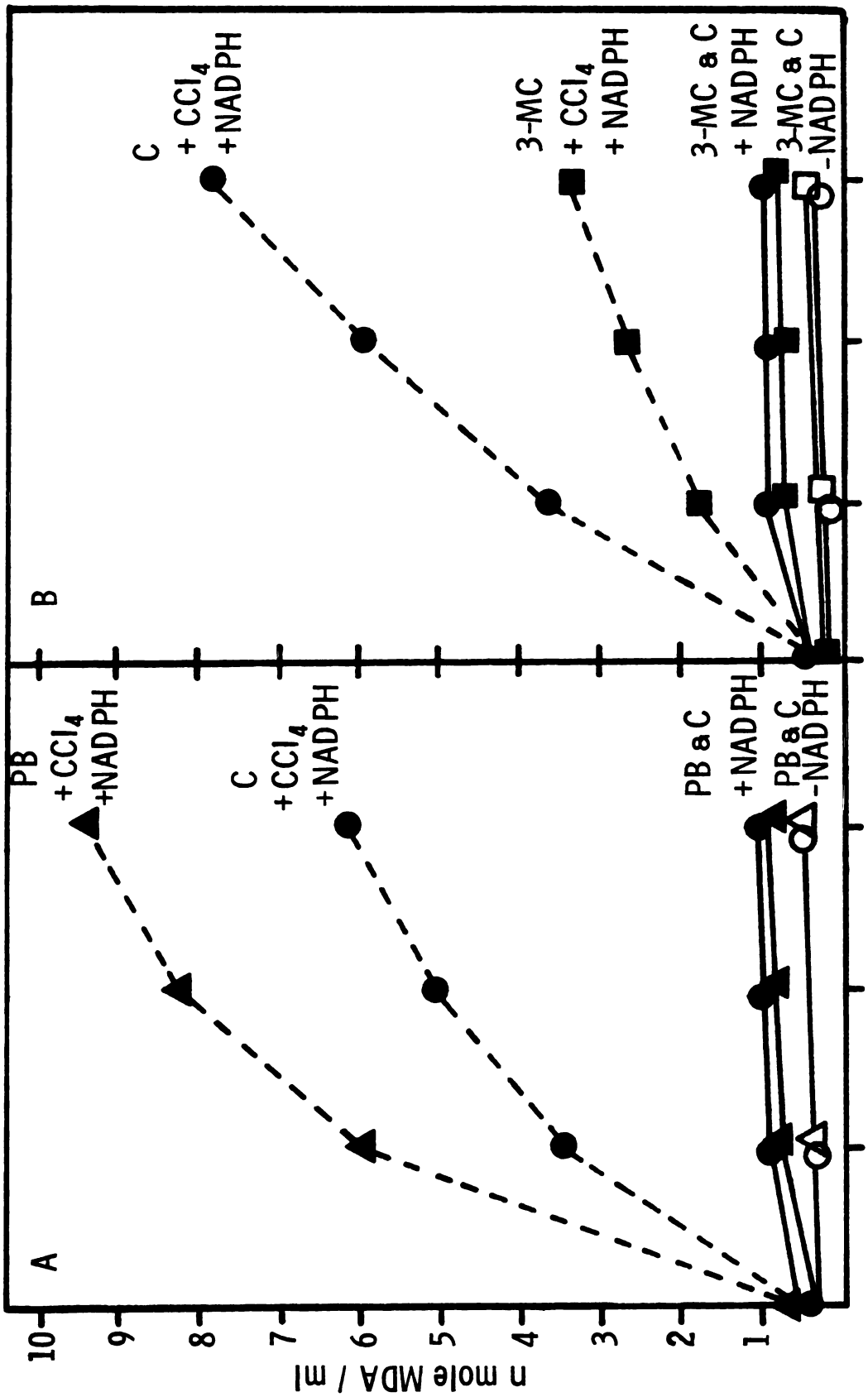

A
B
PB
+CCl4
+NADPH
C
+CCl4
+NADPH
PB & C
+NADPH
PB & C
-NADPH
C
+CCl4
+NADPH
3-MC
+CCl4
+NADPH
3-MC & C
+NADPH
3-MC & C
-NADPH
n mole MDA / ml
Time min
Time min

Fig. 9.

compared to controls. Suarez et al. (1975) have reported similar findings for CCl_4-dependent lipid peroxidation both *in vivo* and *in vitro* when measured by diene conjugation and MDA formation, respectively.

It was of interest that the initial rate of CCl_4-stimulated lipid peroxidation was less in 3-MC-microsomes than in control (PEG)-microsomes even though the cytochrome P_{450} (P_{448}) content was higher, 1.16 and 0.67 nmole cytochrome P_{450} (P_{448})/mg, respectively (Tables 22 and 23). Lipid peroxidation in PB-microsomes was greater than its controls and correlated better with cytochrome P_{450}

TABLE 23.--The Effect of PB- and 3-MC Pretreatments on CCl_4-Dependent Lipid Peroxidation in Rat Liver Microsomes.

The reactions contained 1 mg/ml microsomal protein, ± 1 µl CCl_4/ml, and ± 0.3 mM NADPH in 0.1 M Tris-HCl, pH 7.4 at 37°C. Reactions were conducted as described in Methods and the initial rates of MDA formed expressed either on the basis of protein or cytochrome P_{450} (P_{448}) content.

Pretreatment	CCl_4-Dependent Lipid Peroxidation	
	MDA/5 min/mg	MDA/5 min/nmole Cyt P_{450}
Microsomes		
Control (saline)	2.60	3.44
PB	5.26	1.77
Control (PEG)	2.78	4.17
3-MC	0.88	0.76

contents of 2.97 and 0.76 nmole cytochrome P_{450}/mg, respectively. This relationship does not hold, however, when the rates of CCl_4-dependent lipid peroxidation are compared on the basis of microsomal cytochrome P_{450} content, turnover number (Table 23). The values are much greater in controls than in both PB- and 3-MC-microsomes indicating lipid peroxidation (MDA formation) is more efficient in controls. These results suggest qualitative differences in the cytochrome P_{450} hemoproteins which may be a reflection of their ability to initiate lipid peroxidation as well as function as a pro-oxidant in the propagation reactions, Chapter II.

Because several factors can interfere with microsomal lipid peroxidation, it was of interest to also use CCl_4 binding to microsomal lipids as a measure of CCl_4 activation. Table 24 contains the results of investigations in which the turnover numbers of CCl_4 activation, binding, by the different types of microsomes are compared. Again, the values were similar for the controls but different for PB- and 3-MC-microsomes, suggesting qualitative differences in the ability of cytochrome P_{450} hemoproteins to activate CCl_4. The magnitude of the value for 3-MC-microsomes has shifted and is now intermediate between those for control- and PB-microsomes.

CCl_4 Activation by Reconstituted Mixed-Function Oxidase Systems Containing Cytochrome P_{450} and P_{448} Hemoproteins from PB- and 3-MC-Microsomes

The investigation of CCl_4 activation in microsomes from rats pretreated with various drugs suffers from the possible

TABLE 24.--The Effect of PB- and 3-MC-Pretreatments on $^{14}CCl_4$ Binding to Lipid in Rat Liver Microsomes.

The reactions contained 2.0 mg/ml microsomal protein, 1 µl $^{14}CCl_4$/ml (0.25 mCi/ml CCl_4), 0.1 m EDTA, and ± 0.3 mM NADPH in 0.1 M Tris-HCl, pH 7.4 at 37°C. Reactions were conducted under anaerobic conditions as described in Methods for 5 min at 37°C.

Pretreatment		cpm/nmole P_{450} ± S.D.	Expt'l.-Cont.
Microsomes			
Control (saline)	(-) NADPH	762 ± 15	--
Control (saline)	(+) NADPH	1,537 ± 36	775*
PB	(-) NADPH	193 ± 4	--
PB	(+) NADPH	468 ± 3	275*
Control (PEG)	(-) NADPH	834 ± 36	--
Control (PEG)	(+) NADPH	1,572 ± 68	738*
3-MC	(-) NADPH	480 ± 5	--
3-MC	(+) NADPH	1,099 ± 41	619*

*Significantly different from control, $p < 0.01$.

interference from drug residues as mentioned earlier as well as from other microsomal components. To circumvent these problems the investigation of CCl_4 activation by a reconstituted mixed-function oxidase system was undertaken. Cytochrome P_{450} reductase, cytochrome P_{450}, and cytochrome P_{448} were purified as described in Chapter II. That these hemoproteins were catalytically different is demonstrated in Table 25. This table shows these reconstituted systems' metabolism of benzphetamine and BP, which are substrates specific for the PB- and 3-MC-inducible hemoproteins, respectively. As expected, the relative rates of metabolism of these compounds

TABLE 25.--Benzphetamine and Benzpyrene Metabolism by Reconstituted Mixed-Function Oxidase Systems Containing Cytochromes P_{450} and P_{448} from PB- and 3-MC-Microsomes.

Reactions contained 0.1 mg/ml di-1,2-GPC, 0.20 units/ml NADPH-cytochrome P_{450} reductase, and 0.4 nmole/ml of either PB-P_{450} (90 mM, 9.88 nmole/mg) or 3-MC-P_{448} (90 mM, 7.62 nmole/mg). Other components included and the procedures for conducting the assays have been described in Methods.

Description	Benzphetamine Demethylation (nmole/min/ml)	Benzpyrene Hydroxylation (nmole/min/ml)
Reconstituted Systems		
PB-P_{450}	13.22	0.049
3-MC-P_{448}	2.34	2.43

closely follow that found in the microsomes from which the hemoproteins were isolated. Figure 10 shows a comparison of $^{14}CCl_4$ activation by the two reconstituted systems. The initial rate of activation by the cytochrome P_{448}-containing system is slightly less but not significantly different from that determined for the system containing cytochrome P_{450}. This observation was also found to be consistent with other similar experiments not reported here, but is in contrast to the data obtained from microsomes (Tables 23 and 24). The activation of $^{14}CCl_4$ in a reconstituted system containing equal amounts of mixed-function oxidase components differs from that in microsomes from pretreated and control rats. Such differences may be accounted for if one or more necessary components for CCl_4 activation are limiting, for example, cytochrome P_{450} or

Figure 10.--$^{14}CCl_4$ Activation by Reconstituted Microsomal Mixed-Function Oxidase Systems Containing Cytochromes P_{450} and P_{448}.

Reactions contained 0.1 mg/ml di-1,2,-GPC, 0.20 unit/ml NADPH-cytochrome P_{450} reductase, 0.4 nmole/ml of either cytochrome P_{450} (9.88 nmole/mg) or cytochrome P_{448} (7.62 nmole/mg), 0.1 mM EDTA, 10 mM $MgCl_2$, 0.01% sodium deoxycholate, 1.0 µmole lipid-PO_4/ml, 1 µl $^{14}CCl_4$/ml (0.25 m Ci/ml CCl_4), ± 0.3 mM NADPH in 0.1 M Tris-HCl, pH 7.4 at 37°C. Reactions were conducted under anaerobic conditions as described in Methods at 37°C.

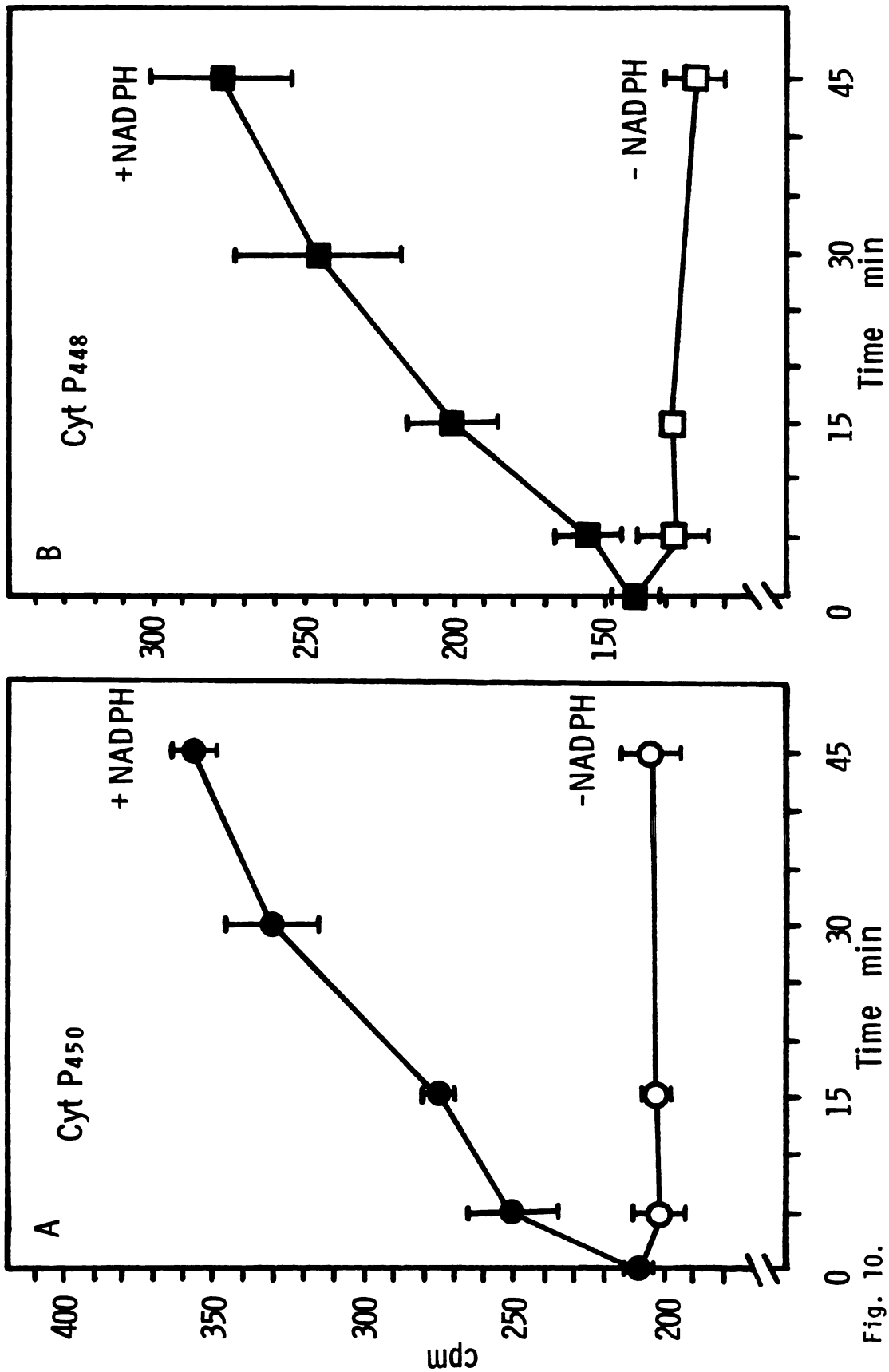

A
Cyt P450
+ NADPH
-NADPH
cpm
400
350
300
250
200
0
15
30
45
Time min
B
Cyt P448
+NADPH
- NADPH
300
250
200
150
0
15
30
45
Time min

Fig. 10.

cytochrome P_{450} reductase, or by interferences from other endogenous microsomal constituents with the method used to measure activation.

Discussion

Substrate specificity of the microsomal mixed-function oxidase system is due to the existence of multiple cytochrome P_{450} hemoproteins. Cytochrome P_{450} activates CCl_4 to its toxic intermediate $\cdot CCl_3$; however, the extent of activation and cytotoxicity does not strictly correlate with cytochrome P_{450} contents of tissues within the same or different animal species (Diaz Gomez et al., 1975; Villarruel et al., 1977). This lack of correlation may be due to differences in substrate specificities of the cytochrome P_{450} hemoproteins. At first glance support is given to this hypothesis by the results of the investigations presented in Tables 23 and 24 and Figure 9. When expressed as nmoles MDA/min/mg protein, the rates of lipid peroxidation in control, PB-, and 3-MC-microsomes, Table 23, parallel those previously reported and correspond to the relative CCl_4 toxicities reported for rats pretreated with these inducers (Castro et al., 1973; Suarez et al., 1972, 1975).

This relationship was not observed when the rates of CCl_4-dependent lipid peroxidation of $^{14}CCl_4$ binding to microsomal lipid were expressed on the basis of microsomal cytochrome P_{450} content, that is, turnover numbers. For example, when measured by either of these methods, the turnover numbers for CCl_4 activation were greater in

control than in PB- or 3-MC- microsomes. The relative turnover number values for 3-MC-microsomes were dependent on the parameter measured (Tables 23 and 24). The value was either greater or lower than that in PB microsomes when $^{14}CCl_4$ binding and CCl_4-stimulated lipid peroxidation, respectively, were used to measure activation. $^{14}CCl_4$ binding to microsomes may be a better indicator of CCl_4 activation in 3-MC-microsomes, since it is a more direct assay and would not be subject to interference from residual 3-MC and its lipophilic metabolites in the membrane. For example, Suarez et al. (1975) demonstrated that 3-MC when added to rat liver microsomes in quantities greater than the residual amounts found after 3-MC-pretreatment of rats would inhibit lipid peroxidation. Though the residual contents of 3-MC and its hydroxylated metabolites in these microsomes were not quantitated, it would provide an explanation for the observed decreases in turnover number (Table 23). The binding assay also would not be affected by the possibility of differences in pro-oxidant or peroxidase activities of cytochromes P_{448} and P_{450}, which could interfere with MDA formation.

The relative values of turnover numbers for control, 3-MC-, and PB-microsomes may be due to factors other than differences in substrate specificities. Although an interaction between CCl_4 and the Type I substrate binding sites on cytochrome P_{450} hemoproteins occurs (Recknagel et al., 1977), it is not a requirement for CCl_4 activation. Activation of CCl_4 by reduced methemoglobin and hemin (Table 18) suggests that interaction with the reduced heme iron is

critical. This leaves open the possibility that differences in rates of cytochrome P_{450} reduction may be the determining factor for rates of CCl_4 activation.

In rat liver microsomes NADPH-cytochrome P_{450} reductase is limited and is usually present in the ratio of one molecule of the enzyme to 20 molecules of cytochrome P_{450} (Peterson et al., 1976). Enzyme induction alters this ratio; for example, the ratio of NADPH-cytochrome P_{450} reductase to cytochrome P_{450} (units/nmole hemoprotein) was calculated from Table 22 to be 0.41, 0.39, 0.23, and 0.15, respectively, for PEG-, saline-, 3-MC- and PB-microsomes. The relative magnitudes of these ratios follow the same order as the turnover numbers determined for $^{14}CCl_4$ binding to microsomal lipids (Table 24). A plausible interpretation of these data is that the relative amounts of NADPH-cytochrome P_{450} reductase, and hence the relative rates of reduction of cytochrome P_{450}, are more important than differences in substrate specificities in determining rates of microsomal CCl_4 activation. Support for this hypothesis comes from the results of the experiments presented in Figure 10. An experiment was conducted in which CCl_4 activation by two reconstituted mixed-function oxidase systems was determined. Each system contained the same amount of hemoprotein, though catalytically different (Table 25), and NADPH-cytochrome P_{450} reductase. The rates of $^{14}CCl_4$ activation did not differ significantly. Differences in rates of reduction of cytochrome P_{450} hemoproteins may also result from the preferential reduction by one or more

NADPH-cytochrome P_{450} reductase enzymes (Coon et al., 1977). Although this is a possibility, evidence in support of this is lacking for rat liver microsomes (Gillette, 1971b; Peterson et al., 1976).

An interesting aspect of the interaction between CCl_4 and reduced cytochrome P_{450} is the formation of CO. Wolf et al. (1977) demonstrated that in the presence of low oxygen concentrations, less than 8%, significant amounts of CO were formed from CCl_4 by either reduced microsomes or purified cytochrome P_{450}. To account for this product a mechanism was proposed which involved briefly the initial formation of a cytochrome $P_{450}(Fe^{+2})$-CCl_4 complex, further reductions and dechlorination to a cytochrome P_{450}-dichlorocarbene complex, and dissociation and subsequent hydrolysis of the carbene to CO. In addition to being hydrolyzed to CO, dichlorocarbene ($:CCl_2$) will add to olefins forming dichlorocyclopropane derivatives (Castro and Kray, 1966; Kleveland et al., 1977). Of importance to the experimental results reported in this chapter were the observed differences in the rates of CO formation in liver microsomes from rats pretreated with PB and BP (Wolf et al., 1977). The cytochrome $P_{448}(Fe^{+2})$-CCl_4 complex in BP-microsomes was less stable than that for PB-microsomes and resulted in a two-fold greater rate of CO, and hence $:CCl_2$, formation. If the same were true for 3-MC-microsomes, which also contain cytochrome P_{448}, the differences in relative magnitudes of turnover numbers for PB- and 3-MC-microsomes observed in Tables 23 and 24 may be rationalized.

The $:CCl_2$ intermediate would be responsible for a portion of the binding observed, whereas the $\cdot CCl_3$ intermediate can bind to microsomal lipid as well as initiate lipid peroxidation. A proportionately greater production of $:CCl_2$ by 3-MC- than by PB-microsomes would result in a higher turnover number for binding (Table 24), but not for lipid peroxidation (Table 23) in 3-MC-microsomes. Identification and quantitation of all by-products of CCl_4 activation by the different cytochrome P_{450} hemoproteins are required to support this hypothesis. The possibility exists that the comparison of $^{14}CCl_4$ binding to microsomal lipid *in vitro* under anaerobic conditions is not the most reliable gauge for predicting CCl_4 toxicity *in vivo*. Toxicity, and hence $\cdot CCl_3$ formation, may therefore be better equated with CCl_4-dependent lipid peroxidation catalyzed by these hemoproteins. Table 23, for example, shows that PB-microsomes are less efficient in CCl_4 activation than control, but because there is more cytochrome P_{450} the overall rate of lipid peroxidation is greater. This correlates well with previous reports of PB-enhanced CCl_4 hepatotoxicity (Recknagel and Glende, 1973; Suarez et al., 1972).

The conclusions to be drawn from the data presented in this chapter are listed below. Microsomes from untreated rats or those pretreated with PB and 3-MC activate CCl_4 to $\cdot CCl_3$ and $:CCl_2$ at different rates. These rates are more dependent on the relative proportions of NADPH-cytochrome P_{450} reductase, and hence the rates of cytochrome P_{450} reduction, than on differences in substrate

specificity. Whether or not CCl_4 will be toxic to a given tissue or animal is dependent on the microsomal cytochrome P_{450} content, its pro-oxidant activity and rate of reduction, the relative amount of the $\cdot CCl_3$ intermediate formed, and the cell's defense mechanisms which include the cellular antioxidant content and peroxidase activities.

CHAPTER V

SUMMARY

The NADPH-cytochrome P_{450} reductase component of the liver microsomal mixed-function oxidase system does not activate CCl_4 to $\cdot CCl_3$. Reaction conditions of a model system containing liposomes and purified NADPH-cytochrome P_{450} reductase were adjusted so that activation by the fully reduced and half-reduced forms could be tested. When investigated under conditions favoring either one or the other enzyme form, neither NADPH oxidation nor the covalent binding of $^{14}CCl_4$ to liposomes occurred.

Although incapable of a direct activation of CCl_4, NADPH-cytochrome P_{450} reductase can participate indirectly in CCl_4 toxification reactions. One reaction involves the pro-oxidant catalyzed degradation of LOOH into free radical intermediates which are capable of CCl_4 activation via atom transfer reactions. In the model system the enzyme reduces both ADP-Fe^{+3} and EDTA-Fe^{+3}. ADP-Fe^{+2} in the presence of oxygen initiates the formation of LOOH whereas EDTA-Fe^{+2} acts as a pro-oxidant. <u>In vivo</u> the pro-oxidant activity is due to cytochromes P_{450} and b_5, other oxidized hemoproteins, hemin, and some transition metals.

The other activation reaction involving NADPH-cytochrome P_{450} reductase is its reduction of cytochrome P_{450} hemoproteins.

These reduced hemoproteins catalyze the rapid activation of CCl_4 via what has been described as electron capture reactions. Dithionite-reduced cytochromes P_{450} and P_{448}, hemin, and methemoglobin also catalyzed CCl_4 activation by this mechanism. Activation via hemin and methemoglobin demonstrated that interaction with the reduced heme and not the substrate binding site was critical for CCl_4 activation. Intermediate products of these reactions have been proposed to include $:CCl_2$ in addition to $\cdot CCl_3$. A schematic which best demonstrates the relationship between the microsomal mixed-function oxidase components and CCl_4 activation is shown in Figure 11. The major route for CCl_4 activation is via its interaction with reduced cytochrome P_{450} hemoproteins. It does not require large quantities of LOOH as substrate nor does it involve the destruction of the hemoprotein which occurs during the prooxidant reactions.

The substrate specificities of different cytochrome P_{450} hemoproteins are not critical in determining the rates of CCl_4 activation. Instead the ratios of NADPH-cytochrome P_{450} reductase to cytochrome P_{450}, and hence the relative rates of reduction, are more important. Cytotoxicity of CCl_4 is due to several factors. The microsomal cytochrome P_{450} content, its rate of reduction, and the relative amount of $\cdot CCl_3$, $:CCl_2$, or other activated products are included among these factors. The prooxidant and peroxidase activities of the microsomal cytochrome P_{450} hemoproteins are also factors controlling peroxidative

Figure 11.--Schematic of Carbon Tetrachloride Activation by the Rat Liver Microsomal Mixed-Function Oxidase System.

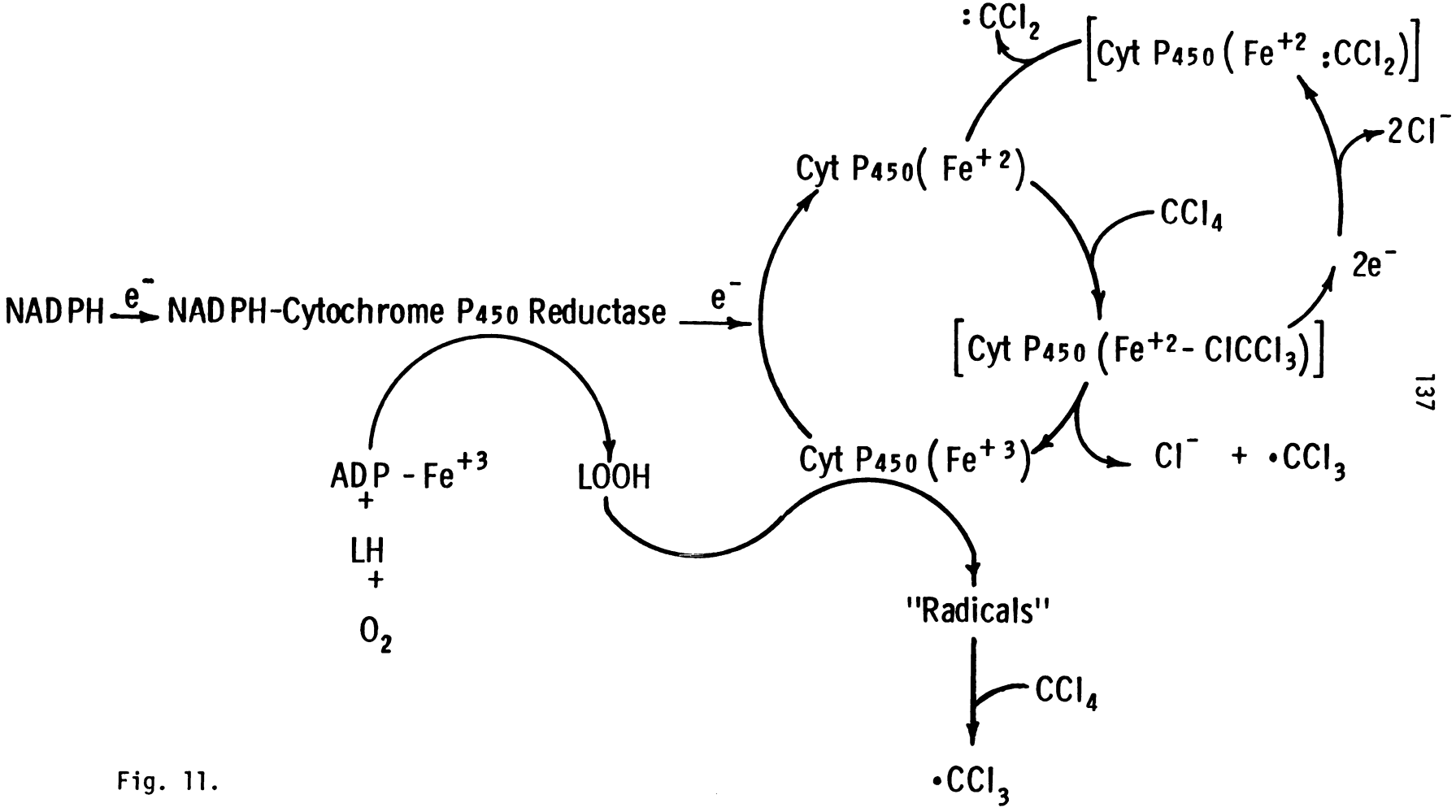

:CCl2
[Cyt P450 (Fe+2 :CCl2)]
2Cl−
Cyt P450(Fe+2)
CCl4
2e−
NADPH e− NADPH-Cytochrome P450 Reductase e−
[Cyt P450 (Fe+2- ClCCl3)]
ADP - Fe+3
+
LH
+
O2
LOOH
Cyt P450 (Fe+3)
Cl− + •CCl3
"Radicals"
CCl4
•CCl3

Fig. 11.

damage to the microsomal membranes and perhaps other cellular components.

Other minor routes of CCl_4 activation that do not involve the microsomal mixed-function oxidase components also occur in the cell. The reduced form of cytochrome b_5 can activate CCl_4 by an electron capture reaction. In vivo this contribution is expected to be of little consequence. Also, a CO-insensitive CCl_4 activation, which is removable by washing, exists in rat liver microsomes. This activity is minor and has not been characterized. It is also possible that ADP-Fe^{+3}, or other weakly chelated iron forms, enhances microsomal CCl_4 activation in vivo as it does in vitro. The causes of this enhancement have not been determined.

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APPENDIX

APPENDIX

Publications

Welton, A. F., O'Neal, F. O., Chaney, L. C., and Aust, S. D. (1975). Journal of Biological Chemistry 250, 5631-5639. "Multiplicity of Cytochrome P_{450} Hemoproteins in Rat Liver Microsomes: Preparation and Specificity of An Antibody to the Hemoprotein Induced by Phenobarbital."

Svingen, B. A., O'Neal, F. O., and Aust, S. D. (1978). Journal of Photochemistry and Photobiology, in press. "The Role of Superoxide and Singlet Oxygen in Lipid Peroxidation."

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