RAT LIVER MICROSOMAL STRUCTURE AND THE MIXED-FUNCTION OXIDASES

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ABSTRACT

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By

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The objectives of this research were: (1) to investigate the general arrangement of proteins within the rat liver endoplasmic reticulum (microsomes), and (2) to investigate the mechanism by which multiple mixed-function oxidase activities are achieved in this membrane. This latter objective was studied by investigating the possibility that multiple forms of NADPH-cytochrome c reductase and cytochrome P_{450} might be present in this membrane and that different forms of these proteins might be inducible by treating rats with phenobarbital and 3-methylcholanthrene, compounds which are known to induce different mixed-function oxidase activities.

The general arrangement of proteins within the microsomal membrane was examined by the techniques of sodium dodecyl sulfate-polyacrylamide gel electrophoresis and lactoperoxidase-catalyzed protein iodination. The membranes used in this study were washed free of ribosomes and adsorbed proteins. Polyacrylamide gel electrophoresis protein profiles indicated that the major protein constituents of the microsomal membrane have molecular weights ranging from 40,000 to

60,000 daltons while the minor protein components have molecular weights ranging between 10,000 to over 200,000 daltons. Enzymatic protein iodination was conducted in the presence of an antioxidant, butylated hydroxytoluene, to prevent the peroxidation of membrane lipids. This procedure preserved the general structure of the membrane during iodination and doubled the incorporation of ^{125}I into membrane proteins. It also prevented the destruction of cytochrome P_{450}. Polyacrylamide gel electrophoresis, following enzymatic iodination, demonstrated that the minor polypeptide components of this membrane, having both low and high molecular weights, and major polypeptide components having molecular weights of approximately 50,000 incorporate ^{125}I and hence are located on the membrane's exterior (cytoplasmic face).

To determine if multiple forms of NADPH-cytochrome c reductase are present in rat liver microsomes, the molecular weights of the detergent-solubilized reductases from control and phenobarbital- or 3-methylcholanthrene-treated rats were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. These enzymes were ^{125}I-labeled in microsomes and then isolated by immunoprecipitation from sodium deoxycholate-solubilized microsomal proteins. The immunoprecipitation was carried out using antibody prepared against a purified, protease-solubilized fragment of NADPH-cytochrome c reductase. The immunoprecipitate was electrophoresed on polyacrylamide gels and the molecular weight of the enzyme determined from the ^{125}I-distribution in the gel. Using this technique the molecular weight of the NADPH-cytochrome c reductases from the liver microsomes of control and phenobarbital or 3-methylcholanthrene-treated rats were each shown to be 79,000 daltons. By this criteria, it was concluded that the

NADPH-cytochrome c reductase enzymes present in all three types of microsomes are identical.

A comparison of the molecular weight of detergent-solubilized NADPH-cytochrome c reductase (79,000) with that of the proteolytically solubilized fragment used for antibody production (71,000) suggests that this enzyme is an amphipathic membrane protein. Thus this protein appears to consist of a single polypeptide chain with a large hydrophilic segment which contains the active site and is exposed to the exterior of the membrane and a smaller hydrophobic segment of approximately 70 amino acids which is buried within the phospholipids of the membrane.

The existence of multiple forms of cytochrome P_{450} was investigated by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis to compare the 40,000 to 60,000 dalton polypeptides present in the rat liver microsomes from control and phenobarbital- or 3-methylcholanthrene treated rats. Since cytochrome P_{450} is thought to be a major microsomal protein having a molecular weight of approximately 50,000 the induction of different forms of this cytochrome by these compounds was observable by this technique. 3-Methylcholanthrene induced a 53,000 dalton protein while phenobarbital induced protein(s) having molecular weights slightly lower than 50,000 daltons. The induced proteins co-purified with cytochrome P_{450} fractions prepared from the three types of microsomes. A method was developed by which benzidine and H_2O_2 could be used to stain for the peroxidase activity of cytochrome P_{420} on polyacrylamide gels. Three hemoproteins were observed in rat liver microsomes using this technique and these had molecular weights of 53,000, 50,000, and 45,000 daltons. 3-Methylcholanthrene induced the 53,000 dalton hemoprotein while phenobarbital induced the 45,000 dalton

hemoprotein. These hemoproteins were also present in partially purified fractions of this cytochrome from the three types of microsomes. These results suggest that multiple cytochrome P_{450} hemoproteins are present in rat liver microsomes.

The spatial position in the microsomal membrane of the hemoproteins induced by phenobarbital and 3-methylcholanthrene was investigated by combining the techniques of sodium dodecyl sulfate-polyacrylamide gel electrophoresis and lactoperoxidase-catalyzed protein iodination. The liver microsomes isolated from rats pretreated with these compounds incorporated more ^{125}I into proteins of approximately 50,000 than did control of microsomes. This suggests that the hemoproteins may be inserted onto the exterior of the microsomal membrane during the induction process.

The 45,000 dalton hemoprotein present in rat liver microsomes was found to be resistant to proteolysis by trypsin and this characteristic was used in its purification. In the procedure used, a sodium cholate-solubilized preparation of cytochrome P_{450} from the liver microsomes of phenobarbital-treated rats was digested with trypsin and the hemoprotein was the purified from proteolytic degradation products by Sephadex G-100 column chromatography. The isolated hemoprotein appeared, on the basis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis, to be homogenous and by spectral assay to be a cytochrome P_{420} hemoprotein. Antibody was prepared against this hemoprotein and immunoprecipitation studies were conducted using detergent-solubilized partially purified cytochrome P_{450} preparations from control and phenobarbital- or 3-methylcholanthrene-treated rats. The antibody specifically immunoprecipitated the 45,000 dalton hemoprotein from

these cytochrome P_{450} preparations. This antibody therefore will be useful in studying the microsomal hydroxylation reactions catalyzed by the 45,000 dalton hemoprotein and in studying the orientation of this protein in the microsomal membrane.

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To My Parents

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I would like to express my sincere appreciation to Dr. Steven D. Aust, my graduate research advisor, for his continual advice, encouragement, and friendship throughout this phase of my scientific career. He has helped me through many tempests both at Michigan State University and on the banks of the Au Sable River. My thanks also go to the members of my guidance committee, Drs. William Wells, Robert Ronzio, John Boezi, and Harold Hafs for their help throughout my stay at Michigan State, and to Dr. Loren Bieber for his assistance in the final phases of the completion of my Ph.D. degree. I have enjoyed collaborative work with many members of Dr. Aust's laboratory both past and present, and these include Dr. David L. Roerig, Dr. Thomas Pederson, Ms. Linda Chaney, Mr. Robert Moore, Mr. John Buege, and Mr. Fred O'Neal. In addition I want to thank the members of the Department of Biochemistry in general for their help and friendship throughout the past five years. The financial assistance of the National Science Foundation and the Department of Biochemistry is gratefully acknowledged.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

Figure Page

ABBREVIATIONS

ADP	adenosine-5'-diphosphate
ATPase	adenosine-5'-triphosphatase
BHT	butylated hydroxytoluene or 2,6-ditert-butylated-cresol
CPM	counts per minute
DEAE	diethylaminoethyl
DDT	1,1,1-trichloro-2,2-tris(p-chlorophenyl)ethane
EDTA	ethylenediaminetetraacetate
IgG	immunoglobulin G
3-MC	3-methylcholanthrene
NADH	reduced nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
PB	phenobarbital
Reductase	NADPH-cytochrome c reductase
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
TBA	thiobarbituric acid
Tris	Tris(hydroxymethyl)aminomethane

INTRODUCTION

The rat liver endoplasmic reticulum (microsomes) serves as the matrix within which are found the enzymes of a mixed-function oxidase system. These enzymes utilize NADPH and O_2 in the hydroxylation of drugs, steroids, fatty acids, and a wide variety of xenobiotics. The work reported in this thesis was initiated upon the premise that a better understanding of the structure of the endoplasmic reticulum would in turn lead to a better understanding of the proteins involved in this electron transport system. Indeed this was thought to be true because components of the mixed-function oxidase system at times constitute nearly 20% of the total microsomal protein.

At the time of initiation of this work, two important techniques in the field of membrane biochemistry had just been developed. These were SDS-polyacrylamide gel electrophoresis and lactoperoxidase-catalyzed iodination of membrane proteins. SDS-polyacrylamide gel electrophoresis for the first time provided a means by which membrane proteins could be completely solubilized and separated from one another. Lactoperoxidase-catalyzed protein iodination provided a mechanism by which only those proteins exposed to the exterior of the membrane could be ^{125}I-labeled, since this enzyme is impermeable to membranes. In combination, these two provided a simple means by which

to study the spatial arrangement of proteins within a membrane. Thus the experimental work presented in the first chapter of this thesis describes the use of these two techniques for studying the topographical arrangement of proteins in the rat liver endoplasmic reticulum.

The remaining chapters of this thesis then describe studies in which these two techniques were used to investigate questions which have been the subject of much current research in the area of microsomal mixed-function oxidases. The mixed-function oxidase system is known to catalyze the hydroxylation of a wide variety of lipophilic compounds with greatly differing structures. Because such apparent nonspecificity is contrary to many of our current theories concerning enzyme catalysis, many investigators have attempted to reconcile this apparent contradictory property by providing evidence for the existence of multiple mixed-function oxidase activities. One of the best arguments for this hypothesis is the fact that different microsomal hydroxylation activities can be induced in rat liver microsomes by pretreating animals with different types of substrates for the mixed-function oxidase system. Thus pretreatment of rats with the compounds phenobarbital (PB) or 3-methylcholanthrene (3-MC) induces different liver microsomal hydroxylation activities. But it is not known how this induction is accomplished at the molecular level. At present the mixed-function oxidase electron transport system is thought to be made up of at least two components, a flavoprotein, NADPH-cytochrome c reductase, and the substrate-binding, terminal oxidase, cytochrome P_{450}. It is possible that different electron transport chains exist in microsomes for each class of substrate hydroxylated and that PB and 3-MC then induce different chains. Indeed spectral and catalytic

evidence already suggests the presence of multiple forms of cytochrome P_{450} in microsomes. But if PB and 3-MC induce different electron transport chains, one would expect that multiple forms of NADPH-cytochrome c reductase should also exist in these microsomes. Since this protein has not been purified from microsomes, however, this is a difficult problem to investigate. By combining the techniques of SDS-polyacrylamide gel electrophoresis and lactoperoxidase-catalyzed protein iodination with various immunochemical methods, I was able to investigate this problem further without purifying this enzyme. In chapter two, evidence is presented to suggest that a single form of this flavoprotein exists in microsomes. This observation then argues against the theory that the different microsomal hydroxylation activities result from multiple electron transport chains.

In light of existing data it would therefore appear that the site of multiplicty must lie in a single component of the electron transport chain. That is, the different hydroxylation activities must be due to the existence of multiple forms of the substrate-binding, terminal cytochrome. Thus in the final two chapters of this thesis, the mechanism of cytochrome P_{450} multiplicity in rat liver microsomes was investigated. While spectrally and catalytically different forms of this cytochrome have been observed in microsomes, there are at least two mechanisms which could explain these observations. Either multiple cytochrome P_{450} hemoproteins are present in microsomes or there is but a single hemoprotein whose spectral and catalytic properties are modified by as of yet unidentified "regulatory" components within the membrane. My work provides evidence for the former mechanism and hopefully future work from Dr. Aust's laboratory will establish the

exact relationship between the multiple cytochrome P_{450} hemoproteins observed in these studies and the different rat liver microsomal mixed-function oxidase activities.

ORGANIZATION OF THE THESIS

Four areas of experimental endeavor are presented in this thesis in the form of separate chapters. Each chapter is organized using a format similar to that used for most scientific papers. That is, each chapter has its own Abstract, Introduction, Materials and Methods, Results, and Discussion sections. The references for each chapter, however, have been combined at the end of the thesis. These chapters are preceded by a literature review which is designed to provide a broad overview of current knowledge on the structure and function of the rat liver endoplasmic reticulum and the microsomal mixed-function oxidases. The introduction to each chapter then provides a more specific background on the subject matter of that chapter.

Also it should be noted that in this thesis when the term protein is used in reference to SDS-polyacrylamide gels it refers to those species which stain with Coomassie blue on the gels. While SDS-gel electrophoresis is known to separate complex proteins into their polypeptide chains, I have made no differentiation between the terms protein and polypeptide in this context because currently there is little information about the arrangement of polypeptide subunits into proteins within membranes.

LITERATURE REVIEW

The Rat Liver Endoplasmic Reticulum

In electron micrographs of cross sections of rat liver parenchymal cells, the endoplasmic reticulum appears as a series of parallel membranes forming tubules, cisternae, and vesicles throughout the cytoplasm of the cell (1,2). By studying serial sections of such cells it has been concluded that the membranes of these various structures are connected with one another and that the endoplasmic reticulum is actually a continuous tubular network of membranes extending throughout the cellular cytoplasm (3,4). Often the membranes of the endoplasmic reticulum appear to be connected with the outer membranes of other subcellular organelles, such as nuclei (5) and peroxisomes (2), suggesting that all these membranes may have a common origin.

Morphologically the endoplasmic reticulum can be divided into two classes: the rough endoplasmic reticulum, so named because ribosomes stud its cytoplasmic surface; and the smooth endoplasmic reticulum, which is free of ribosomes (2,3). When liver tissue is homogenized, the tubules are "pinched off" into sealed vesicles which retain the normal orientation of the membrane (2,3). That is, the outer face of these vesicles is the side normally exposed to the cellular cytoplasm while the luminal contents of the endoplasmic reticulum is preserved within the vesicular cavity. A mixture of rough and smooth endoplasmic

reticulum vesicles can be relatively easily isolated from a liver homogenate by differential centrifugation. These vesicles pellet as the microsomal fraction by centrifugation of a post-mitochondrial supernatant at 105,000 xg for 90 minutes (2,3). If a relatively mild and short homogenization is performed on the liver tissue, there is usually very little contamination of this fraction with other membranes such as plasma membranes, lysosomes, Golgi, and mitochondria (6,7). It is also possible to separate the total microsomal fraction into rough (ribosome-bound) and smooth subfractions. The most common procedure for this separation is to add CsCl to a post-mitochondrial supernatant of a liver homogenate to aggregate rough microsomes (8). This supernatant is then placed over a discontinuous sucrose density gradient and during centrifugation the aggregated rough membranes pellet while the smooth membranes float within the gradient (8).

There have been many attempts to determine how smooth and rough microsomes differ from one another, outside of the fact that ribosomes are attached to the rough membranes. With regards to the chemical composition of the membranes, both fractions appear to have similar phospholipid to protein ratios after ribosomes and adsorbed or luminal proteins are removed from the membranes (9). Both types of membranes also appear to have similar phospholipid compositions but the smooth membranes appear to have relatively higher levels of neutral lipids (9). Some subtle differences have also been reported with regards to the enzymatic contents of these two membrane populations but in general the membranes have strikingly similar enzyme compositions (10-12).

The endoplasmic reticulum of the liver has a wide variety of functions within the cell. One of the most well-known concerns the

synthesis and intracellular transport of proteins, such as albumin, which are destined to be exported to the blood (12,13). In addition, the membrane of this organelle contains two electron transport chains, one involved in the oxidation of NADPH and the other in the oxidation of NADH. The metabolic function of the NADPH-linked sequence is to participate in the hydroxylation of a wide variety of lipophilic compounds (14-16). Two microsomal proteins are known to be associated with this electron transport chain, NADPH-cytochrome c reductase, a flavoprotein (17), and cytochrome P_{450}, a hemoprotein (18). Studies concerning these two proteins constitute a major portion of this thesis and the properties of these two proteins will be described in more detail later. The NADH-linked electron transport chain is thought to be involved in fatty acid desaturation (19,20). The components of this system are the flavoprotein, NADH-cytochrome b_5 reductase (21), the other microsomal hemoprotein, cytochrome b_5 (21,22), and an as of yet unidentified "cyanide sensitive enzyme" (19,20). In this system, electrons are thought to be transferred from NADH to cytochrome b_5 via NADH-cytochrome b_5 reductase. Cytochrome b_5 in turn transfers electrons to the "cyanide-sensitive" enzyme which then catalyzes the desaturation (19,20).

Besides electron transport enzymes, liver microsomes also contain a number of phosphatases, such as glucose-6-phosphatase, which catalyzes the final step in hepatic glycogenolysis (23). In addition, a Mg^{+2}-activated ATPase has been found in rat liver microsomes (24). Enzymatic activities associated with phospholipid biosynthesis (25) and cholesterol biosynthesis (26) have also been localized in the microsomal subfraction.

Very little is known about the specific arrangement of phospholipid and protein constituents within the microsomal membrane. In accordance with current theories of membrane structure (27,28), the phospholipid components of this membrane are most likely arranged in a bilayer which is penetrated to various extents by hydrophobic portions of membrane proteins. Since proteases such as trypsin (29), chymotrypsin (29), and cathepsins (30), have been observed to release catalytically active fragments of specific microsomal proteins, such as NADH-cytochrome b_5 reductase (30), cytochrome b_5 (29), and NADPH-cytochrome c reductase (29), it is assumed that these proteins are located in the outside of the microsomal vesicle. This is true because it has been shown that the proteases cannot diffuse through the microsomal membrane (29,31,32). Recently the "native" forms of two of these proteins, NADH-cytochrome b_5 reductase (33) and cytochrome b_5 (34), have been isolated by Strittmatter and co-workers. Each protein is a single polypeptide chain consisting of a large globular hydrophilic segment, containing the catalytic site, and a smaller hydrophobic polypeptide segment at one end of the protein. It is hypothesized that the hydrophilic portion of each protein is located on the exterior surface of the membrane while the hydrophobic "tail" portion is buried within the phospholipid bilayer of the membrane, functioning to anchor the rest of the protein to the membrane (33,35). But while a great deal is known about the arrangement of NADH-cytochrome b_5 reductase and cytochrome b_5 in the microsomal membrane, these proteins constitute only a small portion of all the microsomal proteins (33,35). Recently techniques have become available for more general investigations of membrane structure and in the first chapter, two of these techniques,

SDS-polyacrylamide gel electrophoresis and lactoperoxidase-catalyzed protein iodination, have been used to more thoroughly examine the arrangement of all the proteins within the rat liver endoplasmic reticulum.

The Microsomal Mixed-Function Oxidase System

As previously discussed, the rat liver endoplasmic reticulum contains an electron transport system which utilizes both NADPH and O_2 in the hydroxylation of various lipophilic compounds (14-16). Because this system utilizes both NADPH and O_2 it is often referred to as the microsomal mixed-function oxidase system (36). Substrates for this system include drugs, carcinogens, pesticides, and many other foreign compounds (xenobiotics) which by various mechanisms end up within the body. The mixed-function oxidase system functions in the removal of these compounds from the body by oxidizing various functional groups of these lipophilic compounds making them more water soluble and hence more easily excreted into the urine, bile or air. Thus this system is usually associated with detoxification, but, ironically, its oxidative reactions have also been implicated in the activation of various toxins, such as carcinogens (37). Endogenous body constituents, such as fatty acids and steroids are also substrates for this system (14-16).

A biochemically unique and somewhat baffling property of the mixed-function oxidase system is its apparent non-specificity with regard to the reactions it catalyzes. For example, it catalyzes such widely diverse reactions as the oxidation of saturated and aromatic compounds, the dealkylation of secondary and tertiary amines, the oxidative cleavage of ethers, the sulfoxidation of thio-ethers, the

epoxidation of aromatic hydrocarbons, halogenated aromatic hydrocarbons, and alkenes, and the conversion of phosphothionates to their phosphate derivatives (38). Brodie et al. (39) observed one unifying property of these reactions, however, in that most of them can be visualized as hydroxylation reactions, which in some instances, form unstable intermediates. Nevertheless, the apparent nonspecificity of this system has been a consideration of much interest to workers in the field of xenobiotic metabolism because such a property is contrary to the common biochemical concept of substrate specificity as found with other enzyme systems.

Another unusual property of the mixed-function oxidase system is the inducibility of its hydroxylation activity by in vivo pretreatment of animals with various lipophilic xenobiotics. This property was first described by Brown et al. (40) in 1954 when it was found that treatment of animals with polycyclic hydrocarbons enhanced the hydroxylation of these compounds by the mixed-function oxidases. Subsequent investigations indicated that this enhanced activity was most probably the result of an increased synthesis of the enzyme constituents of this system (14,41-43). This induction phenomenon is important to pharmacologists since it explains the often observed ability of animals to develop tolerance to certain drugs. Tolerance is developed because the drugs are capable of inducing the enzymes which catalyze their detoxification. While over two hundred drugs, insecticides and carcinogens have been reported to stimulate the mixed-function oxidase system, the inducers can be divided into two classes: general inducers which increase hydroxylation activity towards most substrates and specific inducers which selectively stimulate activity towards

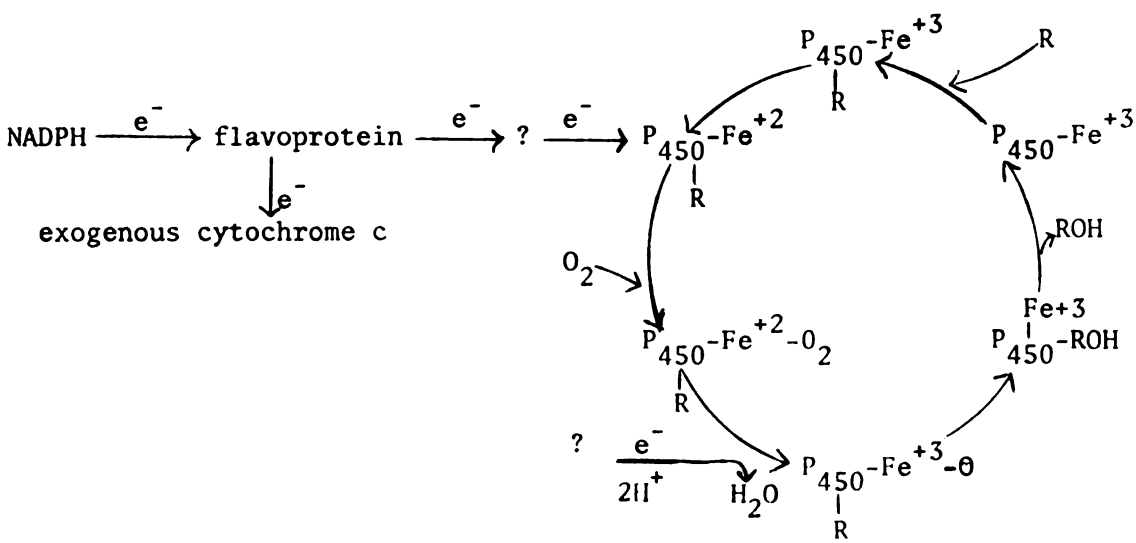
particular substrates only (16). In the laboratory, the induction of the mixed-function oxidase enzymes by phenobarbital (PB), a general inducer, and 3-methylcholanthrene (3-MC), a specific inducer, has been the most widely studied.

The Protein Constituents of the Mixed-Function Oxidase System

The association of the microsomal proteins, NADPH-cytochrome c reductase and cytochrome P_{450}, with the mixed-function oxidase electron transport chain is now well established (14-16). Cytochrome P_{450}, a hemoprotein, is so named because its reduced, CO-difference spectrum exhibits an absorbance maximum at 450 nm. NADPH-cytochrome c reductase, a flavoprotein, derives its name from its ability to transfer electrons from NADPH to exogenous cytochrome c. Pretreatment of animals with the general inducer, PB, induces the levels of both of these proteins in microsomes while pretreatment with the specific inducer, 3-MC, only induces the cytochrome (14-16).

Cytochrome P_{450} was discovered in liver microsomes in 1958 by Klingenberg (44) and Garfinkel (45). It was subsequently characterized as a hemoprotein by Omura and Sato (46,47). Two lines of evidence suggested the involvement of this cytochrome in microsomal hydroxylations. First of all, the levels of this hemoprotein were increased by compounds, such as PB and 3-MC, which were known to induce microsomal hydroxylation activities (48,49). Secondly, Estabrook and co-workers showed that CO inhibited mixed-function oxidase hydroxylations and that the photochemical action spectrum for the reversal of this inhibition was identical to the reduced, CO-difference spectrum of cytochrome P_{450} (50). Because of the requirement for both O_2 and NADPH in

microsomal hydroxylations, it was hypothesized that this microsomal cytochrome reacted with oxygen to form an "active oxygen" intermediate which in turn was reduced by NADPH during the hydroxylation sequence (39). Evidence that NADPH-cytochrome c reductase was the enzyme catalyzing the transfer of electrons from NADPH to cytochrome P_{450} included: the observed increase in the level of this enzyme in microsomes after pretreatment of animals with PB (14), the observation that addition of cytochrome c to microsomes inhibited microsomal hydroxylations (51), the finding that solubilization of this enzyme from microsomes paralleled the loss of hydroxylation activity (52), and the observed ability of antibody prepared to a proteolytically solubilized form of this enzyme to inhibit both cytochrome P_{450} reduction by NADPH and microsomal hydroxylations (53-55). The following is a schematic summary of the current hypothesized mechanism for the involvement of cytochrome P_{450} and NADPH-cytochrome c reductase in the mixed-function oxidase electron transport systems, taken from Gillette et al. (56).



In this system, substrate (R) is hypothesized to combine with the oxidized form of cytochrome P_{450}. This substrate-cytochrome P_{450} complex then is reduced by an electron from NADPH-cytochrome c reductase to form a reduced substrate-cytochrome P_{450} complex. This complex in turn reacts with oxygen to form a substrate-cytochrome P_{450}-oxygen complex. The sequence of events after this stage is still uncertain but it is believed that a second electron reduces the substrate-cytochrome P_{450}-oxygen complex resulting in the formation of an "active oxygen" intermediate which then decomposes to form hydroxylated product and oxidized cytochrome P_{450}. The source of the second electron required in this hydroxylation scheme is at present unclear. It is most likely the NADPH-cytochrome c reductase transfers the electron in this step also. This point will be discussed in more detail below, however.

Attempts at isolating the native forms of either NADPH-cytochrome c reductase or cytochrome P_{450} have been unsuccessful. The reductase appears to be located on the external surface of the microsomal membrane, since treatment of microsomes with proteases which cannot permeate this membrane (29,31,32) readily solubilizes a fragment of the reductase (29). This fragment is enzymatically active with respect to its ability to reduce cytochrome c and has been isolated in several laboratories (57-59). Cytochrome P_{450} appears to be much more intimately associated with the phospholipids of the membrane since solubilization of this cytochrome from the microsomal membrane requires either the use of detergent or treatment of the membrane with phospholipase A (47,60,61). Cytochrome P_{450} is also a very labile hemoprotein and upon perturbation of the microsomal membrane with salts (62),

proteases (29,63), or phospholipases (47,61,64), or upon solubilization of this cytochrome from the membrane with detergents (47,60), it is often converted to a spectrally distinct and catalytically inactive form called cytochrome P_{420}. This cytochrome is so named because the absorbance maxima of its reduced CO-difference spectra is shifted from 450 nm to 420 nm. Neither form of this cytochrome has been purified to homogeneity.

Based upon the fold purifications obtained in isolations of a trypsin-solubilized form of NADPH-cytochrome c reductase (57-59,65) it can be suggested that this enzyme constitutes about 0.6% of the microsomal protein. Partial purification of cytochrome P_{450} has suggested that this hemoprotein appears to have a molecular weight of 50,000 (61,66,67) and, on the basis of its specific activity in microsomes, probably constitutes 4-5% of the microsomal protein in untreated animals and up to 20% of the protein in PB-treated animals (67,68). Thus it appears that during microsomal hydroxylations, one molecule of reductase must reduce a number of cytochrome P_{450} molecules.

One of the major problems in using whole microsomes to study the mixed-function oxidase hydroxylation reactions is that microsomes contain many other enzymatic activities which often interfere. Ultimately it is hoped that both NADPH-cytochrome c reductase and cytochrome P_{450} can be isolated in their native forms and that microsomal hydroxylations can be studied by recombining these proteins to reconstitute hydroxylation activity. Some success at such reconstitution studies has been reported in the laboratories of Coon (69-72) and Lu (73-75). These investigators have succeeded in resolving solubilized microsomes into three fractions, a NADPH-cytochrome c reductase

fraction, a cytochrome P_{450} fraction, and a phospholipid fraction, which when recombined catalyze the hydroxylation of many of the substrates of the mixed-function oxidase system. The phospholipid fraction can be replaced by pure phosphatidylcholine, which appears to be required for the reduction of cytochrome P_{450} by NADPH-cytochrome c reductase (76). The reductase and cytochrome P_{450} fractions used in these experiments are very impure, however, and although this system represents in improvement over microsomes for studying hydroxylation mechanisms, definitive studies will have to await a more refined system.

Because neither NADPH-cytochrome c reductase nor cytochrome P_{450} has been purified to homogeneity and used to reconstitute hydroxylation activity, it is possible that other undiscovered components may also be involved in the mixed-function oxidase electron transport system. In particular, much attention has been given to the possible involvement of other components in the transfer of electrons from NADPH-cytochrome c reductase to cytochrome P_{450} (hence the inclusion of the ? at this step in the reaction scheme presented above). Such a component has been found in at least two other mixed-function oxidase systems--the system found in adrenal cortex mitochondria which is responsible for steroid metabolism (77), and the soluble mixed-function oxidase of Pseudomonas putida which catalyzes the hydroxylation of D-camphor and certain camphor analogues (78). In both cases electron transfer from the reductase to a cytochrome P_{450} occurs via a non-heme iron protein, adrenodoxin in the adrenal system (77) and putidaredoxin in the bacterial system (78). Such a non-heme iron component has not been found in the liver microsomal system. It has recently been reported, however, that an electron acceptor distinct from the

heme-iron atom of liver microsomal cytochrome P_{450} has been found in the partially purified preparation of this cytochrome which is used in the reconstituted system of Coon and co-workers (79). This suggests the possible involvement of a component other than non-heme iron in the liver mixed-function oxidase system. In addition, Pederson et al. (57) have presented evidence for the existence of a microsomal component other than cytochrome P_{450} which appears to be reduced by NADPH-cytochrome c reductase during the NADPH-dependent peroxidation of microsomal lipid. It is possible that this component is also involved in microsomal mixed-function oxidase hydroxylations.

In early studies of the mixed-function oxidase system it was observed that NADH in the presence of saturating concentrations of NADPH increased the activity of the mixed-function oxidase system (80). This observation has led to speculation in the past that components of the NADH-linked fatty acid desaturase electron transport system may also be involved in microsomal hydroxylations, perhaps by providing the second electron to the oxygenated-cytochrome P_{450}-substrate complex (81). Specifically it has been suggested that the transfer of the second electron to the oxygenated-cytochrome P_{450}-substrate complex may be the rate limiting step in the hydroxylation sequence and that cytochrome b_5 may function to transfer this electron from either NADPH-cytochrome c reductase or NADH-cytochrome b_5 reductase to cytochrome P_{450} (81,82). Since the transfer of electrons from NADH to cytochrome b_5 via NADH-cytochrome b_5 reductase is a much more rapid reaction than the transfer of electrons from NADPH to cytochrome b_5 via NADPH-cytochrome c reductase, this hypothesis would explain the observed ability of NADH to increase microsomal hydroxylation activity.

Several lines of evidence have been recently presented which argue against this mechanism, however. First of all, antibodies directed against either NADH-cytochrome b_5 reductase or cytochrome b_5 do not inhibit microsomal hydroxylations (83). Secondly, preparations of NADPH-cytochrome c reductase and cytochrome P_{450} have been prepared free of cytochrome b_5 and will reconstitute microsomal hydroxylation activity, indicating that cytochrome b_5 is not an obligation component of the hydroxylation system (75,84). In fact, addition of cytochrome b_5 to this reconstituted system actually has been observed to inhibit the hydroxylation activity. This inhibition is reversible if NADH-cytochrome b_5 reductase and NADH are then added to the system. Thus, it appears that the best explanation for the synergistic effect of NADH on microsomal hydroxylations is that NADH, via NADH-cytochrome b_5 reductase, functions to keep cytochrome b_5 in a reduced form and hence prevent the transfer of electrons from NADPH to cytochrome b_5. Actually, cytochrome b_5 in the absence of NADH may be acting as an electron sink tunneling electrons away from rather than to cytochrome P_{450}.

Multiplicity of the Microsomal Mixed-Function Oxidase System

As previously stated, one of the most intriguing aspects in the study of the rat liver microsomal mixed-function oxidase electron transport system is the apparent lack of specificity this system displays with regard to the substrates it hydroxylates. Many studies, utilizing microsomal suspensions, have suggested that this property may be explained by the existence of multiple mixed-function oxidase activities. The existence and differential inducibility of these activities would explain the ability of some compounds, such as PB, to

stimulate the hydroxylation of many compounds while others, such as 3-MC, only stimulate the metabolism of a few (16). Investigations in which the kinetics of the metabolism of specific compounds by microsomes have been studied have provided one type of evidence for this idea. For example, Alvares et al. (85) have reported that while pretreatment of rats with both PB and 3-MC increased the V_{max} for microsomal benzpyrene hydroxylation, 3-MC-pretreatment also lowered the K_m for this reaction while PB-pretreatment did not. Such data suggest that 3-MC was inducing a different metabolizing activity than was PB. Wada et al. (86) have shown that if a Lineweaver-Burk plot were made for the hydroxylation of aniline by rat or mouse liver microsomes, a biphasic line resulted which was indicative of a reaction catalyzed by two enzymes having different K_m values. Pretreatment of animals with PB not only increased the V_{max} for this reaction but also appeared to increase the relative amount of the low K_m component. Pederson and Aust (87) observed similar non-linear Lineweaver-Burk plots when studying the metabolism of aminopyrine by the rat liver microsomal mixed-function oxidase system. Their data too suggested that aminopyrine was hydroxylated by two different enzyme systems with two different K_m values. Pretreatment of animals with PB both increased the V_{max} for microsomal aminopyrine demethylation and increased the relative amount of the low K_m component. Pretreatment of rats with 3-MC did not change the V_{max} for aminopyrine demethylation but did cause a relative increase in the amount of the high K_m component.

Studies using various inhibitors of mixed-function oxidases have provided another type of evidence for the existence of multiple microsomal hydroxylating activities. For example, Sladek and

Mannering (88) have reported that the inhibitor SKF-525A inhibited the demethylation of 3-methyl-4-monomethyl-aminoazobenzene in liver microsomes from PB-induced or untreated rats, but not in the microsomes from 3-MC-induced rats. This too suggests that PB and 3-MC induce different mixed-function oxidases. A similar inhibitory pattern with SKF-525A was also observed by Pederson and Aust (87) while studying aminopyrine demethylation in the microsomes from control and PB- or 3-MC-pretreated rats. Aust and Stevens (89) used another inhibitor, DDT, to further investigate the existence of multiple microsomal aminopyrine demethylase activities. DDT is believed to inhibit aminopyrine metabolism because it is an alternate substrate for the microsomal oxidases. A plot of demethylase activity versus DDT concentration was seen to be made up of three linear segments which in the microsomes from control, PB- or 3-MC-induced rats divided total activity into three components, one not inhibited by DDT, one moderately inhibited, and a third which is extremely sensitive to DDT inhibition. These three components were suggested to represent three separate hydroxylation activities. PB-pretreatment appeared to induce the extremely sensitive component while 3-MC induced the component insensitive to DDT inhibition. It should be noted that this was one of the first studies to suggest the existence of at least three microsomal hydroxylating systems instead of two.

Thus, kinetic studies employing the use of various substrates and inhibitors have led to the conclusion that rat liver microsomes appear to contain multiple mixed-function oxidase activities. The question then becomes: How is this multiplicity manifested in microsomes? Are there different electron transport chains for each class of compound to be metabolized or does multiplicity exist somewhere

within the components of the electron transport chain? It would seem unnecessarily redundant to require both a different NADPH-cytochrome c reductase enzyme and a different cytochrome P_{450} hemoprotein for each class of compound, but this question has not yet been adequately answered. The studies presented in the last three chapters of this thesis were designed to further investigate this question.

CHAPTER ONE

THE SPATIAL ARRANGEMENT OF PROTEINS IN THE RAT LIVER ENDOPLASMIC RETICULUM

Abstract

SDS-polyacrylamide gel electrophoresis and lactoperoxidase-catalyzed protein iodination were used to study the topographical arrangement of proteins within the rat liver endoplasmic reticulum (microsomes). The membranes were isolated by differential centrifugation and washed with 0.3 M sucrose containing 0.1 M sodium pyrophosphate, pH 7.5 to remove ribosomes and adsorbed proteins. On the basis of SDS-polyacrylamide gel electrophoretic analyses, rough and smooth microsomal subfractions had identical protein compositions after this treatment, indicating that it was not necessary to subfractionate the total microsomal fraction if the membranes were first washed with sucrose containing sodium pyrophosphate.

Iodination of microsomal membranes was carried out in the presence of butylated hydroxytoluene (BHT), an antioxidant, to avoid the peroxidation of membrane lipids. This procedure not only protected the membranes from lipid peroxidation, but also doubled the incorporation of ^{125}I into membrane proteins. The use of BHT was especially important in studies conducted to determine the effect of iodination on microsomal enzymatic activities, since destruction of cytochrome

P_{450} was shown to be associated with the peroxidation of microsomal lipids. In the presence of BHT, iodination only slightly decreased the level of cytochrome P_{450} and aminopyrine demethylase activity. Cytochrome b_5 and NADPH-cytochrome c reductase were not affected at all, but NADH-ferricyanide reductase was inhibited nearly 100%.

To determine the distribution of ^{125}I into microsomal proteins (and hence which proteins are located in the exterior of the membrane), ^{125}I-labeled microsomes were electrophoresed on SDS-gels. Many minor constituents of this membrane, having both low and high molecular weights, and major polypeptide components having molecular weights of approximately 50,000 were ^{125}I-labeled. Chloroform:methanol (2:1) extraction of lipids from ^{125}I-labeled microsomes, prior to electrophoresis, did not alter this pattern. When lipids were extracted from the membranes prior to iodination, more membrane protein were ^{125}I-labeled, suggesting that in the intact membrane, only proteins located on the exterior of the membrane are substrates for lactoperoxidase. This interpretation was confirmed by studies with trypsin, a protease which cannot permeate the microsomal membrane. Treatment of ^{125}I-labeled microsomes with this protease removed ^{125}I from microsomal proteins again indicating that lactoperoxidase was only iodinating exterior proteins. During experimentation with trypsin it was also observed that if membranes which were iodinated in the absence of BHT were subsequently digested with trypsin, more membrane proteins were susceptible to proteolytic digestion. This further suggested the importance of using BHT during membrane protein iodination since lipid peroxidation, occurring during iodination in the absence of BHT, would otherwise cause extensive breakdown in the structure of the membrane.

The results of these studies suggest that within the cell, many minor protein components of the endoplasmic reticulum membrane are in direct contact with the cellular cytoplasm while many of the major protein components are either buried within the membrane or oriented on its luminal face.

Introduction

Until the late 1960s the study of membrane proteins was retarded by the lack of a convenient method by which they could be separated from one another. Because these proteins are very hydrophobic, even when solubilized from the membrane by most detergents, they tend to aggregate together. The advent of polyacrylamide gel electrophoresis in the presence of the ionic detergent, sodium dodecyl sulfate (SDS), was therefore an important breakthrough in membrane biochemistry. This provided a simple means by which membrane proteins could be solubilized and separated on the basis of their molecular weights and led to the development of other techniques which could be used in combination with SDS-gel electrophoresis to study membrane structure.

One such technique was lactoperoxidase-catalyzed iodination of membrane proteins. This technique was first introduced by Phillips and Morrison (90,91), who used it to study the arrangement of proteins in the human erythrocyte membrane. Lactoperoxidase catalyzes the following reaction at pH 7.4:

$$H_2O_2 + {}^{125}I^- + \text{Protein} \xrightarrow{\text{Lactoperoxidase}} \text{Protein} - {}^{125}I$$

Iodide is incorporated predominantly into tyrosine residues of proteins, however some incorporation into histidine residues can also occur. Iodination of tyrosine occurs in the aromatic ring ortho to the hydroxyl group (92). It was originally reasoned by Phillips and Morrison that since lactoperoxidase iodinates proteins via an enzyme-substrate complex and since this enzyme is impermeable to membranes because of its high molecular weight (78,000), only those proteins exposed to the exterior of a membrane would be ^{125}I-labeled by this enzyme. After iodination, membrane proteins can then be separated by SDS-gel electrophoresis and the position of the exterior proteins determined by fractionating the gel and analyzing the fractions for ^{125}I by gamma counting. This technique has been widely accepted and has been used to study the structure of many membranes including the surface membranes of erythrocytes (90,91,93), lymphocytes (94), blood platelets (95), and mouse fibroblasts (96), and the inner mitochondria membrane (97). In this chapter results will be reported of experiments designed to use SDS-polyacrylamide gel electrophoresis in combination with lactoperoxidase-catalyzed protein iodination to study the spatial position of proteins in the microsomal membrane.

Materials and Methods

Materials

Male Sprague-Dawley rats, weighing between 200 and 250 g were obtained from Spartan Research Animals, Inc., Haslett, Michigan.

The reagents used for electron microscopy (i.e., glutaraldehyde, uranyl acetate, osmium tetroxide, and lead citrate) were obtained from

the Electron Microscope Lab located in the Pesticide Research Center, Michigan State University, East Lansing, Michigan.

Lactoperoxidase, trypsin (type III), β-galactosidase (grade IV), carbonic anhydrase, alcohol dehydrogenase, ribonuclease-A (type III-A), soybean trypsin inhibitor (type I-S), cytochrome c (type VI), and $NADP^+$-isocitrate dehydrogenase (type IV) were obtained from the Sigma Chemical Company, St. Louis, Missouri. Bovine serum albumin (Pentex) was obtained from Miles Lab., Kankakee, Illinois.

Sodium dodecyl sulfate (SDS), butylated hydroxytoluene (BHT), dithiothreitol, ADP (Fermentation grade), EDTA, NADH, NADPH, thiobarbituric acid, nicotinamide, DL-sodium isocitrate, Tris base, and Brillant Blue R (Coomassie blue) were obtained from the Sigma Chemical Company, St. Louis, Missouri. Aminopyrine was purchased from K and K Laboratories, Plainview, New York. 2,4-Pentanedione and dichlorodimethylsilane were obtained from the Aldrich Chemical Co., Milwaukee, Wisconson. Phenobarbital (PB) was obtained from Merck & Co., Inc., Rahway, New Jersey. Trichloroacetic acid, sodium pyrophosphate, and H_2O_2 were obtained from Mallinckrodt, St. Louis, Missouri. Sucrose was obtained from the Swartz/Mann Division of Becton-Dickinson and Company, Orangeburg, New York. All electrophoresis reagents were obtained from Canalco, Inc., Rockville, Maryland. $Na^{125}I$ (carrier-free) was obtained from New England Nuclear, Boston, Massachusetts. All other reagents were analytical grade.

All aqueous solutions were prepared with water which had been distilled and passed through a mixed bed resin ion exchange column.

PB-Pretreatment of Rats

Rats were pretreated by including 0.1% PB in their drinking water for 10 days prior to sacrifice.

Preparation of Microsomal Membranes

Rats were fasted 18 hours before killing by decapitation. The total microsomal fraction was isolated by differential centrifugation as previously described (98). Rough and smooth microsomal subfractions were isolated according to the method of Bergstrand and Dallner (8). In some cases the isolated membranes were stored by suspension in 0.05 M Tris-HCl, pH 7.5 containing 50% glycerol to a protein concentration of approximately 50 mg/ml and freezing at -15°C under N_2 in the presence of 0.01% BHT. Before use the membranes were washed in 0.3 M sucrose containing 0.1 M sodium pyrophosphate, pH 7.5 by suspension with homogenization to a protein concentration of 1-2 mg/ml and centrifugation at 105,000 xg for 90 minutes. All isolation and washing procedures were carried out at 0-4°C.

Enzyme Assays and Analytical Methods

Cytochrome oxidase (99), catalase (100), 5'nucleotidase (101), NADPH-cytochrome c reductase (98), NADH-ferricyanide reductase (30), cytochrome b_5 (46), and cytochrome P_{450} (46) were assayed by previously described techniques. All assays were performed at 25°C.

Microsomal NADPH-dependent hydroxylation activity catalyzed by cytochrome P_{450} was assayed as aminopyrine demethylation by measuring the formation of formaldehyde using the method of Nash (102). The complete details of the aminopyrine demethylase assay have been described by Pederson (98).

Protein was determined by the method of Lowry et al. (103) and standardized with bovine serum albumin using $E_{cm}^{1\%}$ at 280 nm equal to 6.6 (104). RNA was determined by the method of Munroe and Fleck (105). Lactoperoxidase concentrations were determined spectrophotometrically using a millimolar extinction coefficient at 412 nm equal to 114 (91). Hydrogen peroxide concentrations were determined similarly using molar extinction coefficient of 72.4 at 230 nm (91).

Preparation of Samples for Electron Microscopy

At 0-4°C: Membrane suspensions were fixed in 0.1 M sodium phosphate buffer, pH 7.5 containing 2% glutaraldehyde. After 1 hour the membranes were pelleted at 105,000 xg for 90 minutes and washed 3 times in 0.1 M sodium phosphate buffer, pH 7.5. The pellets were then post-fixed in 1% OsO_4 also in 0.1 M sodium phosphate buffer, pH 7.5. After post-fixing the pellets were washed 3 times with 0.1 M sodium phosphate buffer, pH 7.5.

At room temperature: After post-fixing, the pellets were dehydrated in a graded series of ethanol solutions and finally in propylene oxide. Samples were embedded in Epon 812 (70:30 mixture) for sectioning. Sections were then stained with either uranyl acetate or lead citrate.

Electron microscopy was performed using the Phillips EM-100B electron microscope located in the Pesticide Research Center, Michigan State University, East Lansing, Michigan.

Iodination of Microsomes

Sucrose containing sodium pyrophosphate-washed microsomal membrane preparations (usually 2.5 mg of microsomal protein) were suspended to a protein concentration of 0.5 mg/ml in 0.1 M Tris-HCl, pH 7.5 (at 25°C) containing 10^{-6} M KI (2-10 μC $^{125}I^-$/ml), 5 x 10^{-7} M lactoperoxidase, and 0.0001% BHT. The iodination was usually carried out in a five ml volume in a cellulose nitrate centrifuge tube (1" x 3 1/2"). The reaction temperature was kept at 25°C by incubation in a Dubnoff shaker. After a two minute equilibration period, 5 nmoles H_2O_2/ml (10 μl of 0.5 mM H_2O_2/ml) were added at 1 minute intervals over a three minute reaction period. The mixture was then diluted approximately 7-fold with cold 0.1 M Tris-HCl, pH 7.5 (at 25°C) and centrifuged at 105,000 xg for 90 minutes. This centrifugation was performed at 0-4°C. The pelleted microsomes were either suspended in buffer for subsequent use (i.e., enzymatic assay or trypsin digest) or directly prepared for polyacrylamide gel electrophoresis. (For electrophoresis, 2.5 mg of ^{125}I-labeled microsomes were usually suspended in 1 ml of 1% SDS-buffer as described below.)

Assay for Malondialdehyde Levels in Iodinated Microsomes

After iodination, microsomal pellets were resuspended in 0.05 M Tris-HCl, pH 7.5 (at 25°C) to a protein concentration of (0.5 mg/ml). They were then incubated at room temperature with 2 mM ADP and 0.12 mM Fe $(NO_3)_2$ for 2 minutes to decompose all peroxides present in the membranes to malondialdehyde. Malondialdehyde was then measured by assaying the chromogen formation with thiobarbituric acid (TBA) by a method similar to that described by Pederson (98). One ml aliquots

of the membrane suspensions were mixed with 2.0 ml of TBA reagent (0.375% TBA and 15% trichloroacetic acid in 0.25 N HCl). The mixture was heated for 15 minutes in a boiling water bath. After cooling, the assay mixtures were centrifuged at 1000 xg and the absorbance of the supernatant was measured at 535 nm using a Coleman Jr. Spectrophotometer. The content of malondialdehyde was calculated using a standard curve prepared as described by Pederson (98).

Chloroform:Methanol (2:1) Extraction of ^{125}I-Labeled Microsomes

All procedures were performed at room temperature. ^{125}I-labeled, pelleted microsomes (2.5 mg; 740,000 cpm ^{125}I/mg) were homogenized in 3 ml of chloroform:methanol (2:1). This mixture was centrifuged at 1000 xg to pellet the protein residue. The residue was re-extracted with another 3 ml of chloroform:methanol (2:1) and repelleted. The residue was dried under N_2 and resuspended in 1 ml of 1% SDS-buffer for polyacrylamide gel electrophoresis. The chloroform:methanol supernatants were combined and extracted with 0.2 volumes of H_2O. The resulting two phases were separated. The protein residue and upper and lower phases were analyzed for ^{125}I by removing samples for gamma counting.

Iodination of Lipid-Extracted Microsomal Protein

A microsomal pellet (10 mg of protein) was homogenized in 10 ml of chloroform:methanol (2:1). After centrifugation the supernatant was removed and the protein residue re-extracted with an additional 10 ml of chloroform:methanol (2:1). Both extractions were done at room temperature. The protein residue was then dried under N_2 and suspended

in 0.1 M Tris-HCl, pH 7.5 (at 25°C). 2.5 mg of this lipid-extracted microsomal protein was iodinated as described above; however, the sample was continually agitated throughout the reaction to keep it well suspended. To terminate the reaction, the sample was diluted 6-fold with cold 0.1 M Tris-HCl, pH 7.5 (at 25°C) and centrifuged at 10,000 xg in a Sorvall centrifuge (0-4°C). The pellet was then resuspended with homogenization into 1 ml of 1% SDS-buffer of polyacrylamide gel electrophoresis.

Trypsin Treatment of Microsomes

Microsomes were suspended to 6 mg/ml in 0.05 M Tris-HCl, pH 7.5 (at 25°C) containing 1 mM EDTA and 0.005% BHT. Trypsin was added in a ratio of 10 µg trypsin per mg of microsomal protein and this mixture was incubated under N_2 at 25°C for varying lengths of time. No lipid peroxidation occurred during the proteolysis if BHT was present. To terminate the reaction, soybean trypsin inhibitor was added at a ratio of 5 µg of trypsin inhibitor per µg of trypsin. The reaction mixture was then diluted 3-fold with cold 0.05 M Tris-HCl, pH 7.5 (at 25°C) containing 1 mM EDTA and centrifuged at 105,000 xg for 90 minutes at 0-4°C. The supernatants were assayed for protein released from the microsomes. When this proteolysis was performed with ^{125}I-labeled microsomes, the pellets obtained after proteolysis were immediately resuspended in 1% SDS-buffer for polyacrylamide gel electrophoresis.

SDS-Polyacrylamide Gel Electrophoresis

The SDS-polyacrylamide gel electrophoresis technique of Fairbanks, et al. (106) was only slightly modified. In this procedure 1% SDS was included in the electrophoresis buffer and polyacrylamide gels.

Samples (1-5 mg protein/ml) were also prepared in 1% SDS-buffer which in addition contained 7% sucrose, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 40 mM dithiothreitol. They were then heated at 100°C for 15 minutes. After cooling, 10 µg pyronin B tracking dye/ml was added and the samples (usually between 10-50 µl) were applied to 5.6% polyacrylamide gels (5 mm x 100 mm) which had been prepared in tubes coated with dichlorodimethylsilane. The gels were pre-electrophoresed approximately 30 minutes prior to sample application. Electrophoresis was performed at room temperature with a constant voltage gradient of 5 V/cm. The current ranged between 3-4 mA/tube. The running time under these conditions was about 3 1/2 hours. After electrophoresis the gels were placed in 10% trichloroacetic acid overnight. The gels were then stained for protein overnight in a mixture of 0.4% Coomassie blue--10% trichloroacetic acid--33% methanol (107). Destaining was performed for 6 hours against a mixture of 10% trichloroacetic acid and 33% methanol in a diffusion destaining apparatus similar to that marketed by Bio-Rad Laboratories, Richmond, California. The gels were then placed in 10% trichloroacetic acid overnight to completely remove stain from the background of the gels. The protein banding pattern was visualized by scanning at 550 nm in a Gilford spectrophotometer equipped with a gel scanning attachment. After scanning for protein, gels of radioactive samples were fractionated using a Savant Autogel Divider to determine ^{125}I distribution. Usually 6 drop fractions were collected (1.0-1.2 mm of gel) into small disposable glass test tubes and counted on a Nuclear-Chicago gamma spectrometer. Molecular weight markers β-galactosidase (130,000), bovine serum albumin (68,000), carbonic anhydrase (29,000), alcohol dehydrogenase (37,000), trypsin (23,300)

and ribonuclease-A (13,700) were run in parallel with membrane protein samples. The molecular weights reported in these studies were then calculated as described by Weber and Osborn (108) from a standard curve plotted using these standards. Each value is the average of at least 3 determinations.

Results

Enzymatic and Electron Microsocopic Characterization of the Microsomal Fraction Isolated by Differential Centrifugation

The subcellcular fraction used in these studies was isolated from a rat liver homogenate by differential centrifugation of a post-mitochondrial supernatant at 105,000 xg for 90 minutes. This fraction has previously been characterized by many workers as being derived from the endoplasmic reticulum (2,3). To determine the purity of the membranes used in these studies, the specific activity of various subcellcular organelle marker enzymes in the isolated fraction was compared to their specific activity in the total liver homogenate. The marker enzymes assayed were cytochrome oxidase for mitochondria (99), catalase for peroxisomes (100), NADPH-cytochrome c reductase for microsomes (109), and 5'nucleotidase for plasma membranes (101). It can be seen from Table I that the membrane fraction used in these studies was enriched 8-fold with respect to the microsomal enzyme, NADPH-cytochrome c reductase, while the specific activity of all other marker enzymes was much lower than that of the liver homogenate. This enzymatic comparison confirmed that the membrane fraction used in these studies was indeed enriched in endoplasmic reticulum. In addition,

TABLE I

SPECIFIC ACTIVITY OF ORGANELLE MARKER ENZYMES IN THE MICROSOMAL FRACTION RELATIVE TO THAT OF THE TOTAL LIVER HOMOGENATE

Perfused rat livers were homogenized in 1.15% KCl--0.2% nicotinamide. The homogenate was centifuged at 15,000 xg for 20 minutes to pellet mitochondria, lysosomes, nuclei, peroxisomes, plasma membranes, and cellcular debris. The post-mitochondrial supernatant was then centrifuged at 105,000 xg for 90 minutes to pellet the microsomal fraction.

Assays were performed as described in the Materials and Methods section. Relative specific activity refers to the ratio of the specific activity of each enzyme in the microsomal fraction to its specific activity in the total homogenate.

Enzyme	Relative Specific Activity
Catalase	0.53
Cytochrome oxidase	0.04
NADPH-cytochrome c reductase	8.10
5'-nucleotidase	0.57

electron microscopic examination of this subfraction confirmed this finding in that the fraction appeared to be composed of the small vesicular membrane sacs characteristics of microsomes (2,3) and appeared to be free of other subcellular organelles such as mitochondria, nuclei, lysozomes and peroxisomes (Figure 1).

Comparison of Rough and Smooth Microsomal Membranes After Removal of Ribosomes and Adsorbed Proteins

The total microsomal fraction is known to be a mixture of rough (ribosome-bound) and smooth microsomes and to be contaminated by adsorbed cytoplasmic proteins. Since these studies were only to be concerned with the membrane proteins, it was desirable to establish a procedure by which ribosomes and contaminating cytoplasmic proteins could be removed from the membranes. Previous investigations have indicated that chelating agents are effective in disrupting and removing ribosomes (110-112). In this work a mixture of 0.3 M sucrose containing 0.1 M sodium pyrophosphate, pH 7.5 was used to remove both ribosomes and adsorbed proteins. As can be seen from Table II, this procedure removed approximately 30% of the protein and 80% of the RNA from the membranes. The washing procedure did not appear to be removing membrane proteins since the specific activity of such membrane-associated proteins as NADPH-cytochrome c reductase, NADH-ferricyanide reductase, cytochrome b_5, and cytochrome P_{450} increased. Also the recovery of these proteins were nearly 100%. In addition, the ability of this washing procedure to remove 90% of the catalase from the membrane accentuates its effectiveness at removing adsorbed proteins. This was especially important for studies using

Figure 1. ELECTRON MICROGRAPH OF MICROSOMAL MEMBRANES

Rat liver microsomes were fixed in 0.1 M sodium phosphate buffer, pH 7.5 containing 2% glutaraldehyde and post-fixed in 0.1 M sodium phosphate, pH 7.4 containing 1% OsO_4. They were then embedded in Epon 812 for sectioning. The sections were stained with uranyl acetate and lead citrate. This micrograph was taken using a Phillips EM-100B electron microscope. Magnification: 35,000x.

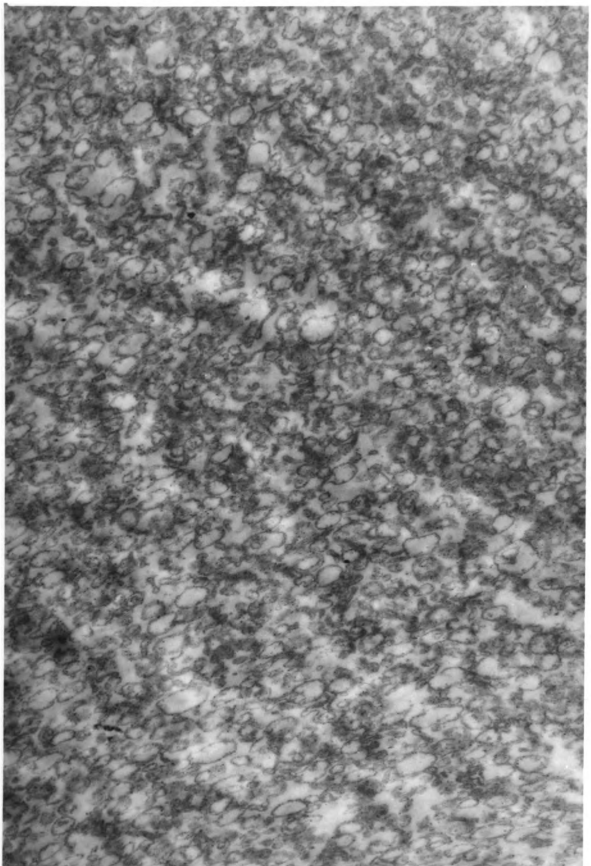


Figure 1

TABLE II

THE LEVELS OF VARIOUS CONSTITUENTS IN THE TOTAL MICROSOMAL FRACTION ISOLATED FROM CONTROL RATS BEFORE AND AFTER WASHING THE MEMBRANES WITH 0.3 M SUCROSE CONTAINING 0.1 M SODIUM PYROPHOSPHATE, pH 7.5

After initially assaying the microsomes, 25 mg of microsomal protein was resuspended to a protein concentration of 1 mg/ml in sucrose containing sodium pyrophosphate. This suspension was centrifuged at 105,000 xg for 90 minutes to pellet the microsomal membrane. The pellet was then resuspended to a protein concentration of 4.5 mg/ml for assay (18.0 mg of protein was found in the pellet resulting in a recovery of 72% of the original protein).

Constituent	Specific Activity		% Recovery
	Before	After	
NADPH-cytochrome c reductase*	0.160	0.224	102
NADH-ferricyanide reductase*	4.30	5.61	93
Catalase**	97.0	10.2	7.6
Cytochrome b_5***	0.423	0.658	112
Cytochrome P_{450}***	0.809	1.28	114
RNA+	107.0	32.4	21.9

*μmoles of receptor reduced/min per mg protein

**μmoles of H_2O_2 consumed/min per mg protein

***nmoles of cytochrome/mg protein

+ng of RNA/mg protein

lactoperoxidase-catalyzed protein iodination since catalase would interfere with the iodination because H_2O_2 is a substrate for both enzymes.

The SDS-polyacrylamide gel electrophoresis profiles of rough and smooth microsomes were similar after washing the membranes with sucrose and sodium pyrophosphate. This can be seen in Figure 2 which compares the electrophoresis profiles characteristics of rough, smooth, and total microsomes before and after washing. The major polypeptide constituents for each type of membrane have molecular weights between 40,000 and 60,000 daltons. Unwashed rough microsomes, however, appear to contain several components of molecular weight above 100,000 and below 40,000 which are not present in smooth microsomes. Since Dice and Schimke (113) have reported that the majority of the ribosomal polypeptides have molecular weights between 15,000 and 34,000 on SDS-gels, the ribosomes may be the source of the lower molecular weight components in rough microsomes. After washing, the profiles obtained for rough and smooth microsomes are identical and resemble those obtained for the total microsomal fraction after washing. This suggests that the membranes have similar protein compositions after being washed free of ribosomes and adsorbed proteins. Such a conclusion is consistent with several other recent investigations of the protein components of rough and smooth microsomes which have been made using polyacrylamide gel electrophoresis (60,67,114,115). On this basis we concluded that for these studies it did not appear necessary to subfractionate the total microsome fraction if the membranes were first washed with sucrose containing sodium pyrophosphate.

Figure 2. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROTEIN PROFILES OF ROUGH, SMOOTH, AND TOTAL LIVER MICROSOMAL FRACTIONS BEFORE AND AFTER WASHING THE MEMBRANES WITH SUCROSE CONTAINING SODIUM PYROPHOSPHATE

The membrane fractions were isolated from a control rat as described in the Materials and Methods section. The upper scans are of gels run on unwashed membranes while the lower scans are of washed membranes. Between 40 to 45 µg of protein was applied to each gel.

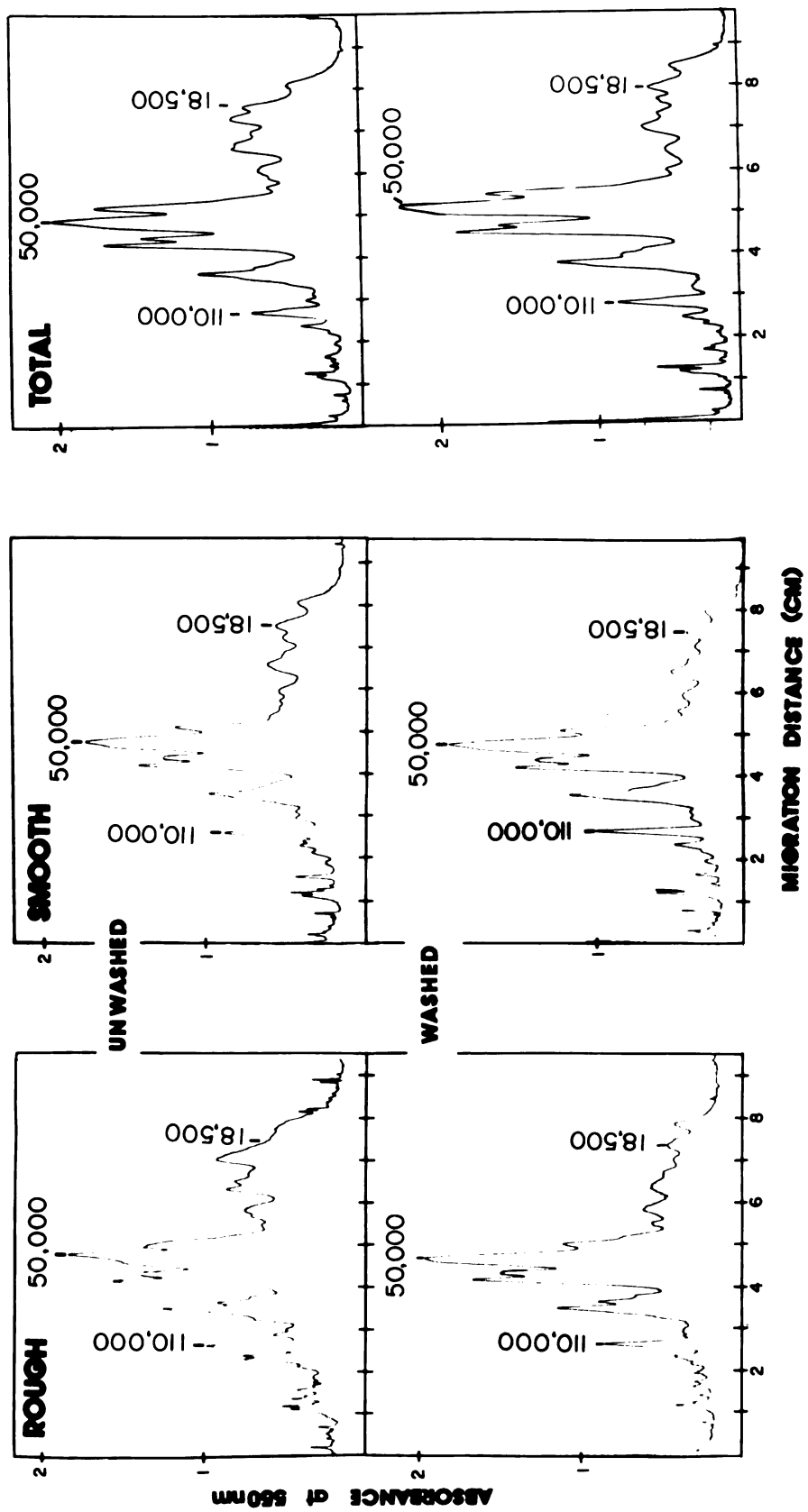

ROUGH
SMOOTH
TOTAL
UNWASHED
WASHED
50,000
110,000
18,500
ABSORBANCE at 550nm
MIGRATION DISTANCE (CM)

Figure 2

For convenience, the initial experiments conducted to adapt the lactoperoxidase-catalyzed iodination technique for use with rat liver microsomes were performed using the microsomes from PB-pretreated rats. PB-pretreatment causes a proliferation of the liver endoplasmic reticulum (14) and therefore large amounts of microsomes could be isolated at one time.

Lipid Peroxidation During Enzymatic Iodination of Rat Liver Microsomes

Phillips and Morrison (90,91) foresaw one potential problem in using the lactoperoxidase-catalyzed iodination technique. It required exposing membranes to H_2O_2 which at the time was thought to be involved in the peroxidative breakdown of membrane lipids. Lipid peroxidation has been associated with the increased permeability of membranes including those of red blood cells (116-118) and such subcellular organelles as mitochondria (119-121), microsomes (122), and lysosomes (123,124). Therefore it was thought that this could be a major problem if one were trying to ^{125}I-label only those proteins located on the exterior of a membrane. To solve this problem, Phillips and Morrison suggested that the concentration of H_2O_2 to which the membranes are exposed be kept low by use of multiple additions of a small amount of H_2O_2 (8 nmole/ml reaction) periodically throughout the course of the iodination. In initial experiments I used a similar technique, adding small aliquots of H_2O_2 (5 nmole/ml reaction) to the iodination mixture at one minute intervals over a three minute reaction period. To determine if lipid peroxidation were occurring during the iodination, malondialdehyde levels were measured in the microsomes after iodination. Malondialdehyde is a breakdown product commonly used to measure the

peroxidation of unsaturated fatty acids (125). As can be seen in Table III, when iodination of rat liver endoplasmic reticulum was carried out using multiple additions of a small amount of H_2O_2, lipid peroxidation still occurred. Iodination in the presence of several agents known to inhibit lipid peroxidation either by chelating iron (EDTA) or acting as antioxidants (dithiothreitol or BHT), indicated that a very low concentration of BHT combined the desirable effects of completely inhibiting lipid peroxidation while doubling the amount of ^{125}I incorporation into microsomes during the iodination. The mechanism by which inhibition of lipid peroxidation leads to an increase in the level of ^{125}I incorporated is unknown but is currently under investigation in Dr. Aust's laboratory. It is now known that H_2O_2 actually does not promote the peroxidation of membrane lipids (98) therefore implying that the lipid peroxidation observed in these experiments may be catalyzed enzymatically by lactoperoxidase.

Determination of Optimum H_2O_2 and Lactoperoxidase Concentrations for Maximum ^{125}I Incorporation into Microsomes

Having determined the proper conditions for inhibiting microsomal lipid peroxidation during an iodination, studies were then conducted to determine the optimum concentrations of H_2O_2 and lactoperoxidase to use during an iodination. The results of these experiments are shown in Figures 3 and 4. On the basis of these results, iodinations were carried out using 5×10^{-7} M lactoperoxidase and 5 nmole H_2O_2/ml aliquot additions of H_2O_2 at one minute intervals over a three minute reaction period.

TABLE III

THE EFFECT OF INHIBITORS OF LIPID PEROXIDATION ON THE FORMATION OF MALONDIALDEHYDE AND INCORPORATION OF ^{125}I INTO MICROSOMES

Liver microsomes from a PB-pretreated rat were washed in 0.3 M sucrose containing 0.1 M sodium pyrophosphate, pH 7.5. They were then resuspended to a concentration of 0.5 mg/ml in 0.1 M Tris-HCl, pH 7.5 (at 25°C) containing 10^{-6} M KI (1 μC $^{125}I^-$/ml), 5 x 10^{-7} M lactoperoxidase, and the additions indicated below. After a two minute equilibration at 25°C, 5 nmole H_2O_2/ml were added at 1 minute intervals over a three minute reaction period. The reaction was terminated by diluation in cold 0.1 M Tris-HCl, pH 7.5 (at 25°C) and centrifugation at 105,000 xg for 90 minutes at 0-4°C. The pelleted microsomes were resuspended in 0.05 M Tris-HCl, pH 7.5 (at 25°C). ^{125}I Incorporation was assayed by removing an aliquot for gamma counting. Malondialdehyde content was assayed as described in the Materials and Methods.

Additions	Malondialdehyde formation nmoles/mg protein	^{125}I-Incorporation Total cpm/mg protein
None	22.0	800,000
40 mM Dithiothreitol	1.3	0
2 mM EDTA	6.1	860,000
0.0001% BHT	0.0	1,610,000

Figure 3. INCORPORATION OF ^{125}I INTO LIVER MICROSOMES FROM A PB-PRETREATED RAT USING VARYING CONCENTRATIONS OF H_2O_2

Sucrose and sodium pyrophosphate-washed microsomes were suspended to a concentration of 0.5 mg/ml in 0.1 M Tris-HCl, pH 7.5 containing 10^{-6} M KI (2 μC $^{125}I^-$/ml), 5 x 10^{-7}M lactoperoxidase, and 0.0001% BHT. After pre-incubation at 25°C for 2 minutes, iodination was initiated by adding the indicated amounts of H_2O_2/ml reaction at one minute intervals over a three minute reaction period. The reaction was terminated by dilution with 0.1 M Tris-HCl, pH 7.5 and centrifugation at 160,000 xg for 90 minutes at 0-4°C. The pelleted microsomes were counted on a Nuclear-Chicago gamma spectrometer to determine ^{125}I incorporation.

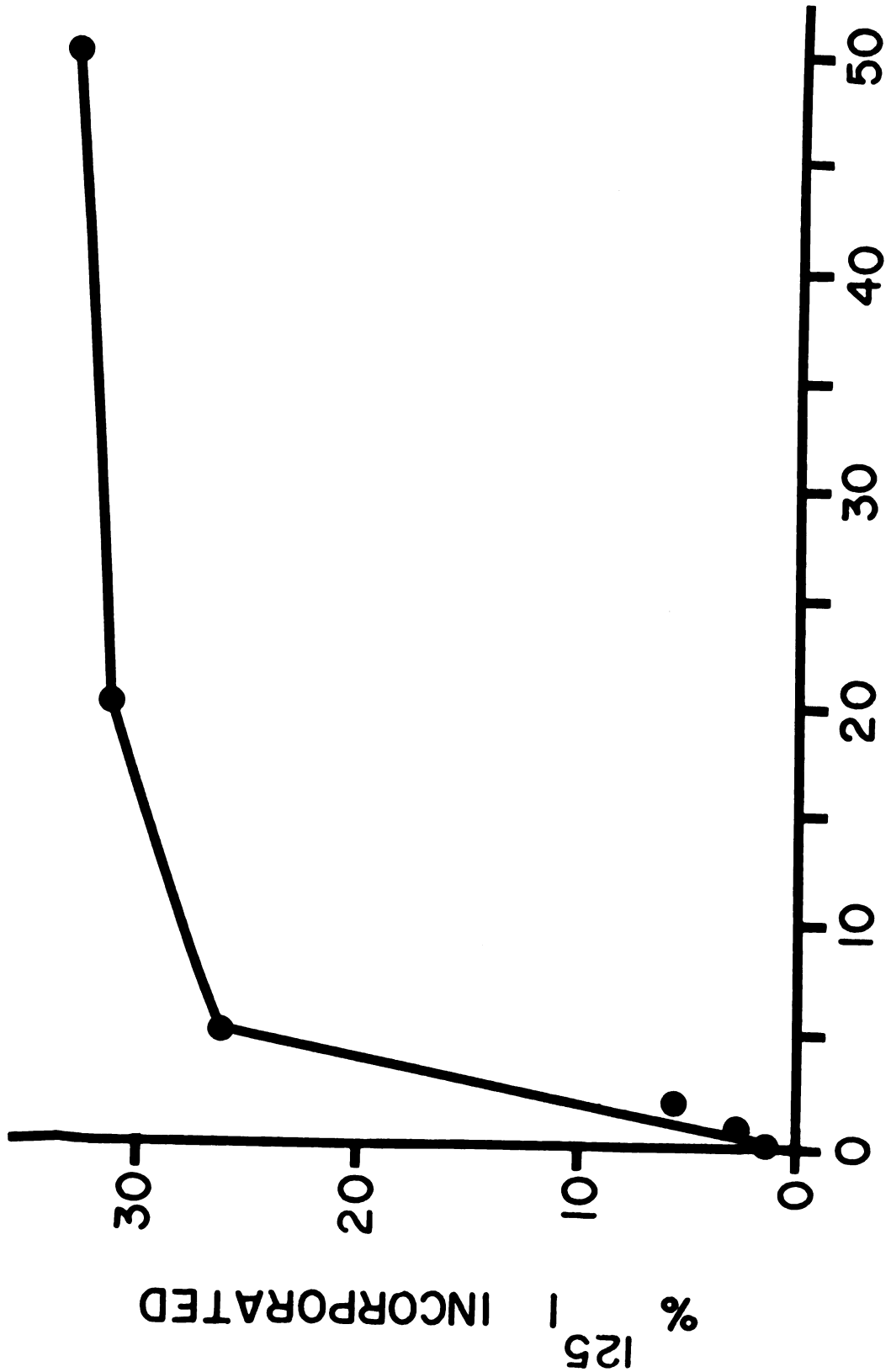


Figure 3

Figure 4. INCORPORATION OF ^{125}I INTO LIVER MICROSOMES FROM A PB-PRETREATED RAT USING VARYING LACTOPEROXIDASE CONCENTRATIONS

Sucrose and sodium pyrophosphate-washed microsomes were resuspended to a concentration of 0.5 mg/ml in 0.1 M Tris-HCl, pH 7.5 containing 10^{-6} M KI (2 μC ^{125}I/ml), varying concentrations of lactoperoxidase, and 0.0001% BHT. After pre-incubation at 25°C for 2 minutes, iodination was initiated by adding 5 nmoles H_2O_2/ml reaction at one minute intervals over a three minute reaction period. The reaction was terminated by dilution with 0.1 M Tris-HCl, pH 7.5 and centrifugation at 160,000 xg for 90 minutes at 0-4°C. The pelleted microsomes were counted on a Nuclear-Chicago gamma spectrometer to determine the amount of ^{125}I incorporated.

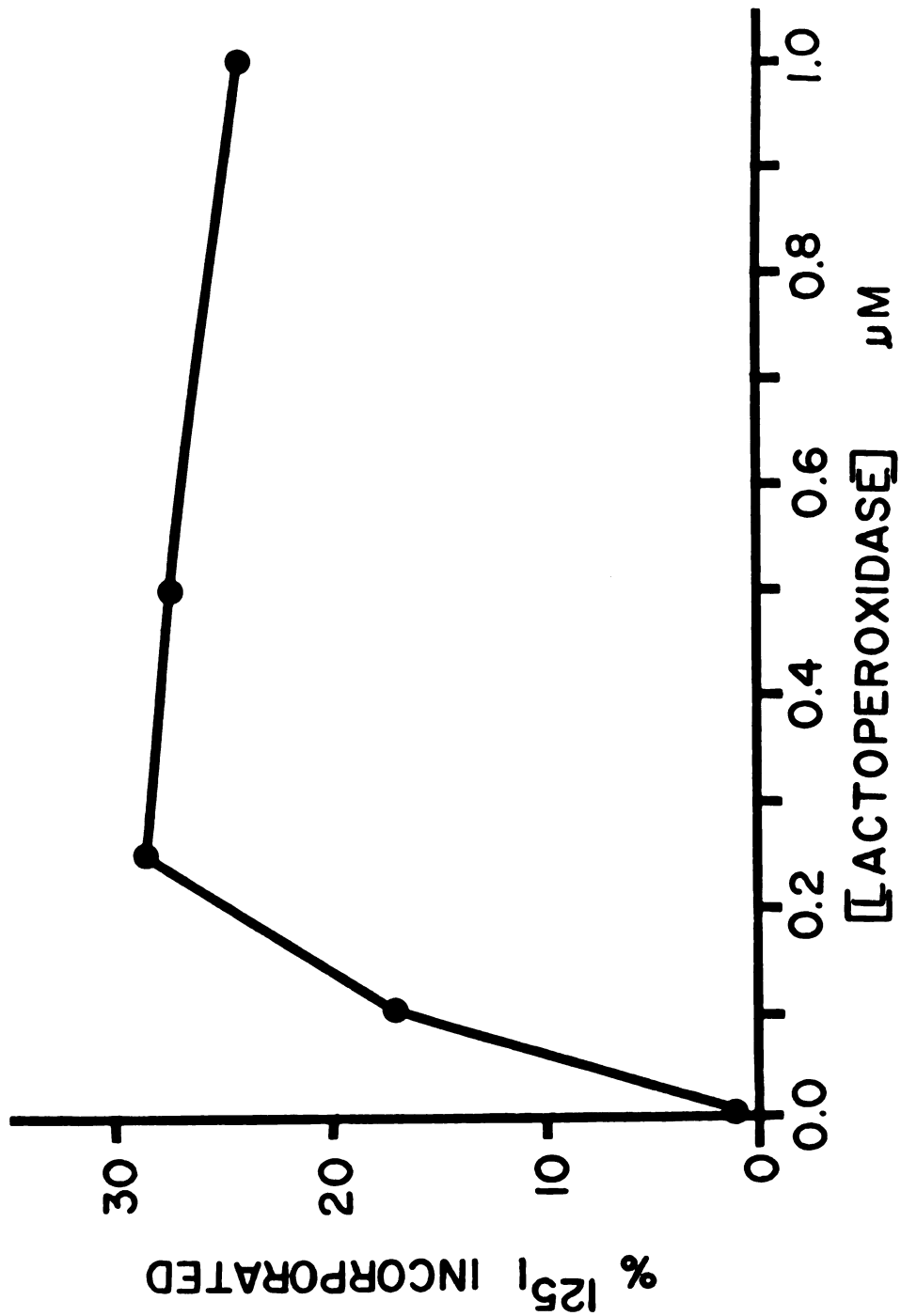

% ^{125}I INCORPORATED
30
20
10
0
0.0
0.2
0.4
0.6
0.8
1.0
[LACTOPEROXIDASE] μM

Figure 4

The Effect of Iodination on Microsomal Cytochromes and Enzymatic Activities Associated with the Mixed-Function Oxidases

Because the technique of lactoperoxidase-catalyzed protein iodination was to be used in future experiments to study the proteins of the mixed-function oxidase system, it was of interest to determine the effect which iodination had on the enzymatic activities associated with these proteins. Since lipid peroxidation has been reported to cause inactivations of membrane-bound enzymes and the destruction of cytochromes (126), it was important to iodinate the membranes in the presence of BHT for these studies. Indeed, data presented in Table IV suggests that there is a correlation between lipid peroxidation and loss of microsomal cytochrome P_{450}. Microsomes incubated aerobically at 25°C showed an increasing malondialdehyde content and decreasing concentration of cytochrome P_{450} with time. Under conditions where lipid peroxidation did not occur, however, cytochrome P_{450} was not destroyed by the aerobic incubation. Other investigators have recently found a similar correlation between cytochrome P_{450} destruction and lipid peroxidation and are currently investigating the mechanism involved (127-129).

In Table V, the effects of iodination on microsomal cytochromes and enzymatic activities are shown. Again a comparison of samples 4 and 5 in this table demonstrates that if iodination had not been carried out in the presence of BHT, erroneous interpretations of the effects of iodination on cytochrome P_{450} levels and aminopyrine demethylase activity would have results. In the absence of lipid peroxidation, iodination still appears to lower cytochrome P_{450} levels

TABLE IV

THE CORRELATION BETWEEN LOSS OF CYTOCHROME P_{450} AND THE PEROXIDATION OF MICROSOMAL LIPID

Liver microsomes from a PB-pretreated rat were washed in 0.3 M sucrose containing 0.1 M sodium pyrophosphate, pH 7.5, suspended to a protein concentration of 5 mg/ml in 0.2 M Tris-HCl, pH 7.5, and aerobically incubated at 37°C. Samples were removed at various times and made 0.005% with BHT to stop lipid peroxidation before assaying. To one sample 0.005% BHT was added initially and this sample was incubated in parallel at 37°C for 90 minutes.

Time incubated (min)	Malondialdehyde nmoles/mg	P_{450} nmoles/mg
5	2.0	2.1
30	7.8	1.7
60	14.0	1.3
90	16.4	0.4
90 + BHT	0.5	2.0

TABLE V

EFFECT OF IODINATION ON LIPID PEROXIDATION, CYTOCHROMES, AND ENZYMATIC ACTIVITIES IN THE LIVER MICROSOMES FROM PB-PRETREATED RATS

Rat liver microsomes from PB-pretreated rats were washed in 0.3 M sucrose containing 0.1 M sodium pyrophosphate, pH 7.5. They were then resuspended and incubated for 5 minutes under the following conditions, centrifuged at 105,000 xg for 90 minutes, and resuspended in 0.05 M Tris-HCl, pH 7.5 (at 25°C) for assays.

1. Microsomes (0.5 mg/ml) incubated at 25°C in 0.1 M Tris, pH 7.5 (at 25°C).
2. Microsomes (0.5 mg/ml) incubated at 25°C in 0.1 M Tris, pH 7.5 (at 25°C) containing 0.0001% BHT.
3. Microsomes iodinated as described in the Materials and Methods + 0.0001% BHT but without KI.
4. Microsomes iodinated as described in the Materials and Methods + 0.0001% BHT.
5. Microsomes iodinated as described in the Materials and Methods -0.0001% BHT.

#	Lipid Peroxidation[a]	P_{450}[b]	b_5[b]	NADPH-cytochrome c reductase[c]	NADH-ferricyanide reductase[c]	Aminopyrine demethylase[d]
1	4.5	1.21	0.68	0.24	4.50	7.5
2	0	2.28	0.75	0.33	5.10	9.7
3	0	2.15	0.83	0.31	4.72	10.3
4	0	1.86	0.97	0.28	0.15	6.7
5	10	0.64	0.86	0.26	0.16	4.1

[a] nmoles malondialdehyde/mg microsomal protein.

[b] nmoles/mg microsomal protein.

[c] µmoles/min per mg microsomal protein.

[d] nmoles formaldehyde/min per mg protein.

by 15% and aminopyrine demethylase activity by 35%. Because cytochrome P_{450} is affected by the iodination, this protein may be located on the exterior of the membrane. This idea is explored more thoroughly in chapter three. Aminopyrine demethylase activity is a measure of the activity of the entire mixed-function oxidase electron transport chain. Since NADPH-cytochrome c reductase activity does not appear to be inhibited, loss of reductase activity could not account for the decreased demethylation activity. Therefore the decrease in the demethylation activity probably results from the loss of cytochrome P_{450} in the microsomes.

Of all the enzymes examined, iodination most significantly affected NADH-ferricyanide reductase activity suggesting that this enzyme (also termed NADH-cytochrome b_5 reductase) is located on the external surface of the microsomes. This would correlate with previous observations which also suggest an exterior location, since this enzyme can be proteolytically cleaved from the membrane by cathepsins (30). Also Strittmatter and co-workers (33) have recently presented evidence to suggest that binding sites for this enzyme are on the external surface of the microsomes. This enzyme contains a tyrosine residue at its active site (130) and iodination of this tyrosine by lactoperoxidase may have caused the observed inhibition.

^{125}I-Labeling Pattern for the Proteins From the Microsomes of a PB-Pretreated Rat

When washed microsomes isolated from a PB-pretreated rat were ^{125}I-labeled using lactoperoxidase and then electrophoresed on SDS-polyacrylamide gels, the ^{125}I incorporation pattern seen in Figure 5

Figure 5. SDS-POLYARCYLAMIDE GEL ELECTROPHORESIS PROTEIN AND ^{125}I-INCORPORATION PROFILES OBTAINED FOR THE LIVER MICROSOMAL FRACTION FROM A PB-PRETREATED RAT

The rat liver microsomal fraction was isolated from a PB-pretreated rat by differential centrifugation and washed with sucrose containing sodium pyrophosphate. The membranes were then iodinated as described in the Materials and Methods. The iodination reaction contained 2 μC $^{125}I^-$/ml. After centrifugation the membranes were resuspended in 1% SDS-buffer for electrophoresis. 40 μg of protein was applied to the gel. The smooth line represents the protein profile obtained by scanning the gel at 550 nm following Coomassie blue staining. The dotted line designates the ^{125}I-incorporation pattern obtained by fractioning the gel and counting the fractions for ^{125}I using a gamma spectrometer.

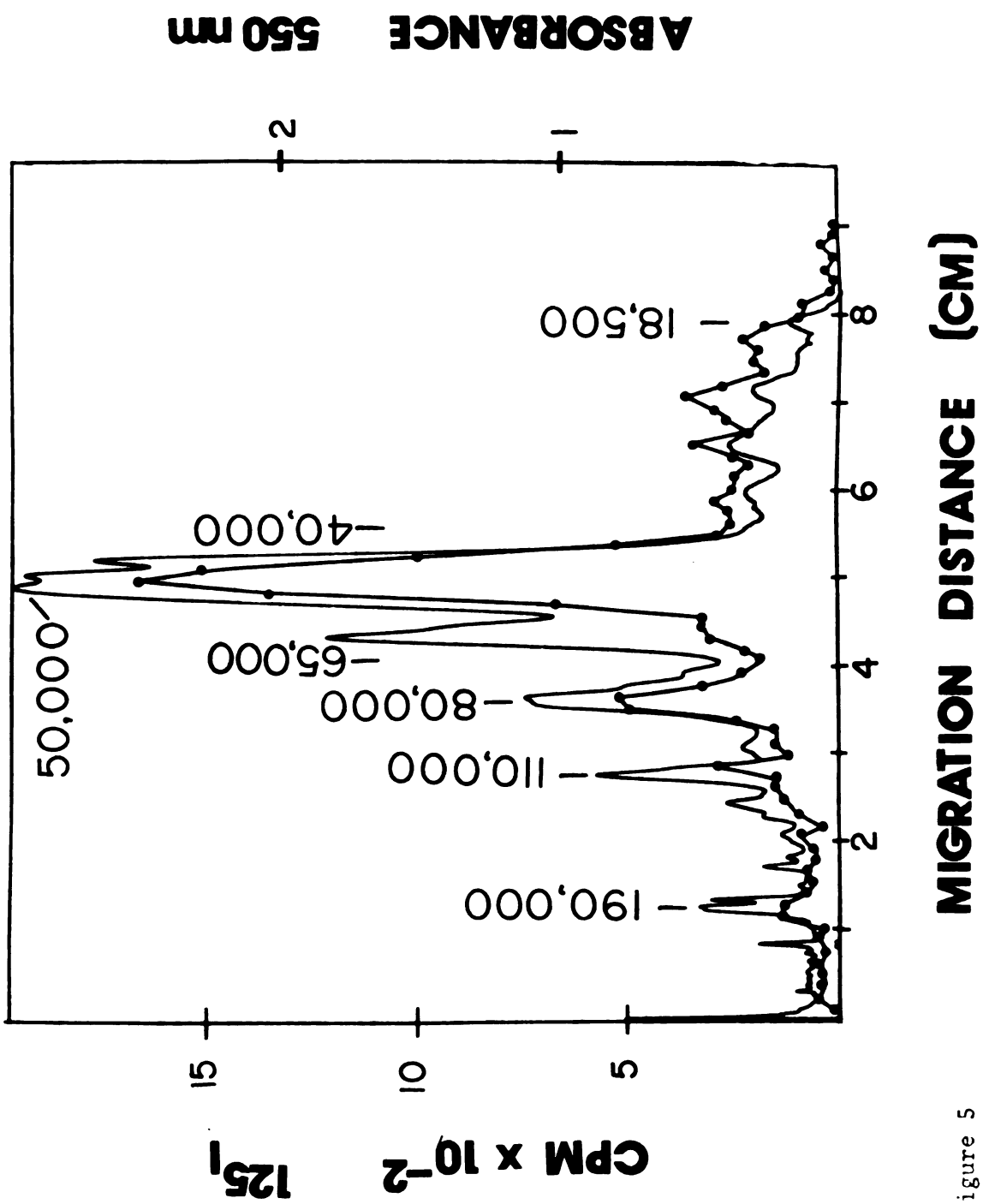

ABSORBANCE 550 nm
CPM x 10⁻² ¹²⁵I
MIGRATION DISTANCE (CM)
190,000
110,000
80,000
65,000
40,000
18,500
50,000

Figure 5

was obtained. This pattern was very reproducible from preparation to preparation of microsomes. From this pattern it appears as if many of the minor protein constituents of microsomes are capable of being ^{125}I-labeled and hence located on the membrane exterior. Also major protein constituents having molecular weights of approximately 50,000 incorporate ^{125}I and therefore are exposed to the membrane's exterior. This ^{125}I-labeling pattern does not change when the specific activity of ^{125}I in the iodination mixture is increased (Figure 6) and therefore it reflects the true distribution of I^- into the membrane proteins. Also if ^{125}I-labeled microsomes are extracted with chloroform:methanol (2:1) to remove lipid and the lipid-depleted microsomal proteins then electrophoresed on SDS-gels, the same protein and ^{125}I-labeling pattern is obtained (Figure 7). This indicates that membrane lipids are not interfering with either pattern.

Recovery of ^{125}I from SDS-Polyacrylamide Gels

When the recovery of ^{125}I from SDS-gels was first examined, it seemed extremely low. Only 20-25% of the original cpm of ^{125}I applied to the gels were recovered in the fractions obtained from the gel. Since no cpm of ^{125}I were found at the top of the gel, this did not appear to be a problem resulting from the inability of some of the membrane proteins to enter the gel. Because the gels were being fractionated after being stained with Coomassie blue, it was initially thought that the radio-activity might be lost from the gels during the staining and destaining process. This could not completely account for the low recovery, however, since the recovery was still low (33%) even if the gel was fractionated immediately after electrophoresis.

Figure 6. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROTEIN AND ^{125}I-INCORPORATION PROFILES OBTAINED FROM THE LIVER MICROSOMAL FRACTION FROM A PB-PRETREATED RAT WHEN THE SPECIFIC ACTIVITY OF ^{125}I IN THE IODINATION REACTION WAS VARIED

Membranes were treated as described in the legend to Figure 5. The upper profile is the protein pattern obtained by scanning gels at 550 nm following Coomassie blue staining. The lower scans are the ^{125}I-incorporation profiles obtained when the membranes were enzymatically iodinated in the presence of 2 μC ^{125}I/ml reaction (▲-▲), 6 μC ^{125}I/ml reaction (■-■), and 10 μC ^{125}I/ml reaction (-●---●-). 40 μg of protein was applied to each gel.

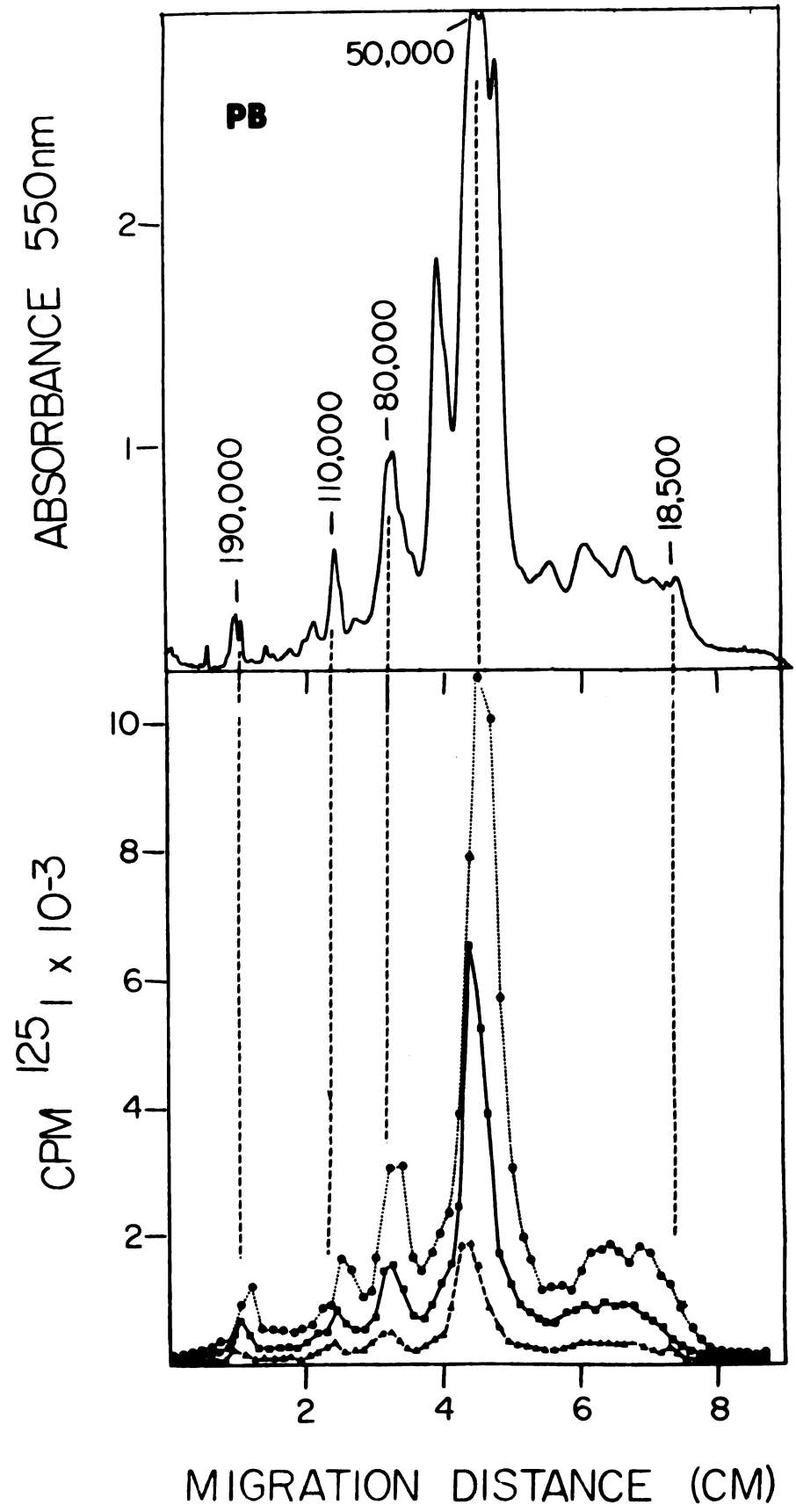

PB
50,000
190,000
110,000
80,000
18,500
ABSORBANCE 550nm
CPM 125I x 10-3
MIGRATION DISTANCE (CM)

Figure 6

Figure 7. A COMPARISON OF THE SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROTEIN AND ^{125}I-INCORPORATION PROFILES OBTAINED BY ELECTROPHORESING ^{125}I-LABELED MICROSOMES BEFORE AND AFTER LIPID EXTRACTION WITH CHLOROFORM:METHANOL (2:1)

Duplicate samples of sucrose containing sodium pyrophosphate-washed liver microsomes from a PB-pretreated rat were iodinated in the presence of 2 μC ^{125}I$^-$/ml reaction as described in the Materials and Methods. After centrifugation, one pellet was immediately suspended in 1% SDS-buffer for electrophoresis. The upper left-hand gel scan represents the protein banding pattern obtained for this sample while the lower left-hand scan is the ^{125}I-incorporation pattern. The second pellet was extracted twice with a mixture of chloroform:methanol (2:1), as described in the Materials and Methods. The protein residue remaining after this extraction was resuspended in 1% SDS-buffer for electrophoresis. The upper scan on the right-hand side is the protein banding pattern obtained for this sample while the lower scan is the ^{125}I-incorporation profile. 40 μg of protein was applied to each gel.

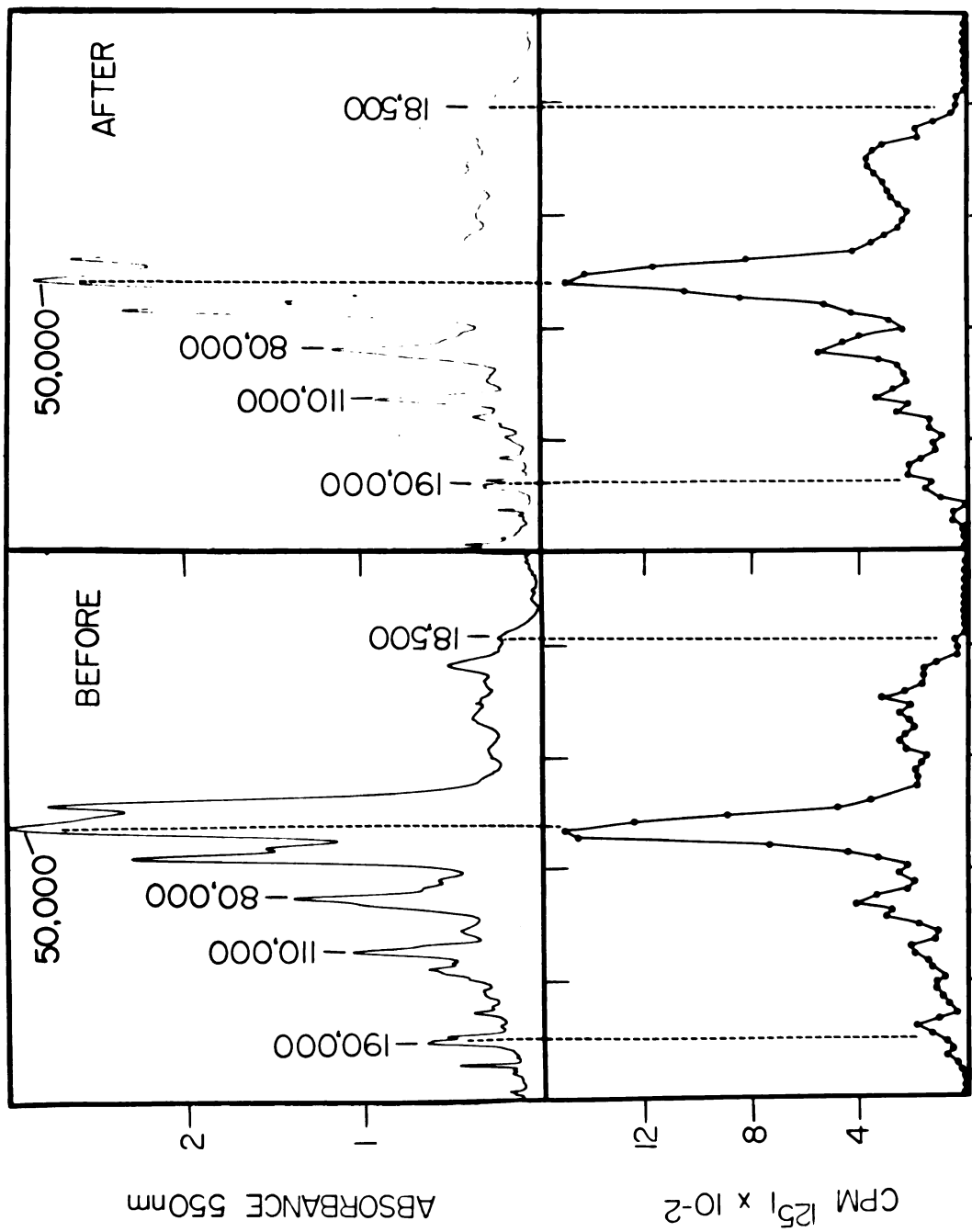

BEFORE
AFTER
50,000
190,000
110,000
80,000
18,500
ABSORBANCE 550nm
CPM ^{125}I x 10-2
MIGRATION DISTANCE (CM)

Figure 7

Furthermore since the ^{125}I-incorporation patterns were identical before or after staining, it did not appear to matter when the gels were fractionated.

To further examine this problem, ^{125}I-labeled microsomes were extracted with chloroform:methanol (2:1) to determine if microsomal lipid might be incorporating the ^{125}I. The results of this experiment are shown in Table VI and demonstrate that, at most, only 11% of the incorporated ^{125}I is found in the lipid. Most of the ^{125}I is recovered in the aqueous phase. The ^{125}I in the aqueous phase is probably not due to the presence of ^{125}I-labeled proteins because the ^{125}I-labeled material could not be precipitated with 10% trichloroacetic acid. It is also probably not free $^{125}I^-$ because of the results obtained from experiments in which controls were run to determine the amount of free $^{125}I^-$ which non-specifically pelleted with microsomes (see Figures 3 and 4). In these experiments, microsomes were incubated under the conditions used for iodination, however either lactoperoxidase or H_2O_2 was excluded from the reaction. In these cases less than 5% of the $^{125}I^-$ normally incorporated into microsomes was found in the pellet. This suggests that the level of non-specific adsorption of free $^{125}I^-$ to the membranes is low. In any case, the incorporation of ^{125}I into a water-soluble molecule which pellets with the microsomal fraction accounts for the apparent poor recovery of ^{125}I from the SDS-gels. Since some RNA is still associated with the microsomal fraction even after the membranes are washed with sucrose containing sodium pyrophosphate (see Table II), this RNA may have been iodinated by lactoperoxidase. Alternatively, glycogen is known to pellet with the microsomal fraction from livers (7). Even though the rats used to prepare

TABLE VI

DISTRIBUTION OF ^{125}I IN FRACTIONS RESULTING FROM A CHLOROFORM:METHANOL (2:1) EXTRACTION OF ^{125}I-LABELED LIVER MICROSOMES FROM A PB-PRETREATED RAT

2.5 mg of ^{125}I-labeled pelleted liver microsomes from a PB-pretreated rat (1,860,000) were homogenized in 3 ml of chloroform:methanol (2:1). This mixture was centrifuged to pellet the protein residue. The chloroform:methanol layer was removed and the residue was re-extracted with another 3 ml of chloroform:methanol (2:1). The chloroform:methanol layer from the two extractions were combined and extracted with 0.2 volume of H_2O. The resulting two phases were separated and analyzed for ^{125}I. The total recovery of ^{125}I in the various fractions was 96%.

Fraction	% of Recovered cpm
Upper phase (acqueous)	49%
Lower phase (organic)	11%
Precipitated protein	40%

microsomes for these studies were starved prior to isolation, some glycogen may still have remained in the livers and contaminated the microsomal subfraction. This then may have been iodinated. These possibilities were not explored further since the lactoperoxidase-catalyzed iodination technique could still be used as a tool to study the arrangement of proteins within the microsomal membrane even though other contaminants, pelleting with these membranes, were also being iodinated.

To determine the actual recovery of ^{125}I from membrane proteins after SDS-gel electrophoresis, the protein precipitate remaining after a chloroform:methanol extraction of ^{125}I-labeled microsomes was solubilized in SDS and electrophoresed. In this case, the recovery of ^{125}I from a stained gel was approximately 70%.

Iodination of Microsomal Protein Extracted with Chloroform:Methanol (2:1)

In studies employing lactoperoxidase to examine the topography of a membrane, it is important to demonstrate that only exterior proteins are being iodinated. To investigate this point, a comparison was made of the iodination patterns of microsomal membranes and lipid-extracted microsomal proteins. If the iodination is specific for proteins on the exterior of the membrane, more membrane proteins should be labeled after lipid extraction. As can be seen in Figure 8, this is the case. After lipid extraction, the overall level of ^{125}I incorporation increases and many more proteins having molecular weights between 40,000 and 60,000 daltons are ^{125}I-labeled.

Figure 8. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROTEIN AND ^{125}I-INCORPORATION PROFILES FOR RAT LIVER MICROSOMES WHICH WERE IODINATED BEFORE AND AFTER EXTRACTING LIPIDS FROM THE MEMBRANES

One sample of liver microsomes from a PB-pretreated rat was iodinated after being washed with sucrose-containing sodium pyrophosphate. A second sample was iodinated under identical conditions but after being washed and subsequently extracted with a mixture of chloroform:methanol (2:1), as described in the Materials and Methods. The iodination reaction in each case contained 2 μC ^{125}I/ml. After iodination these samples were suspended in 1% SDS-buffer for electrophoresis. The protein banding pattern for both samples was identical and is represented by the upper scan. The lower scans represent the ^{125}I-incorporation patterns for microsomes iodinated before lipid extration (▪-▪) and after lipid extraction (-●---●-). 40 μg of protein was applied to each gel.

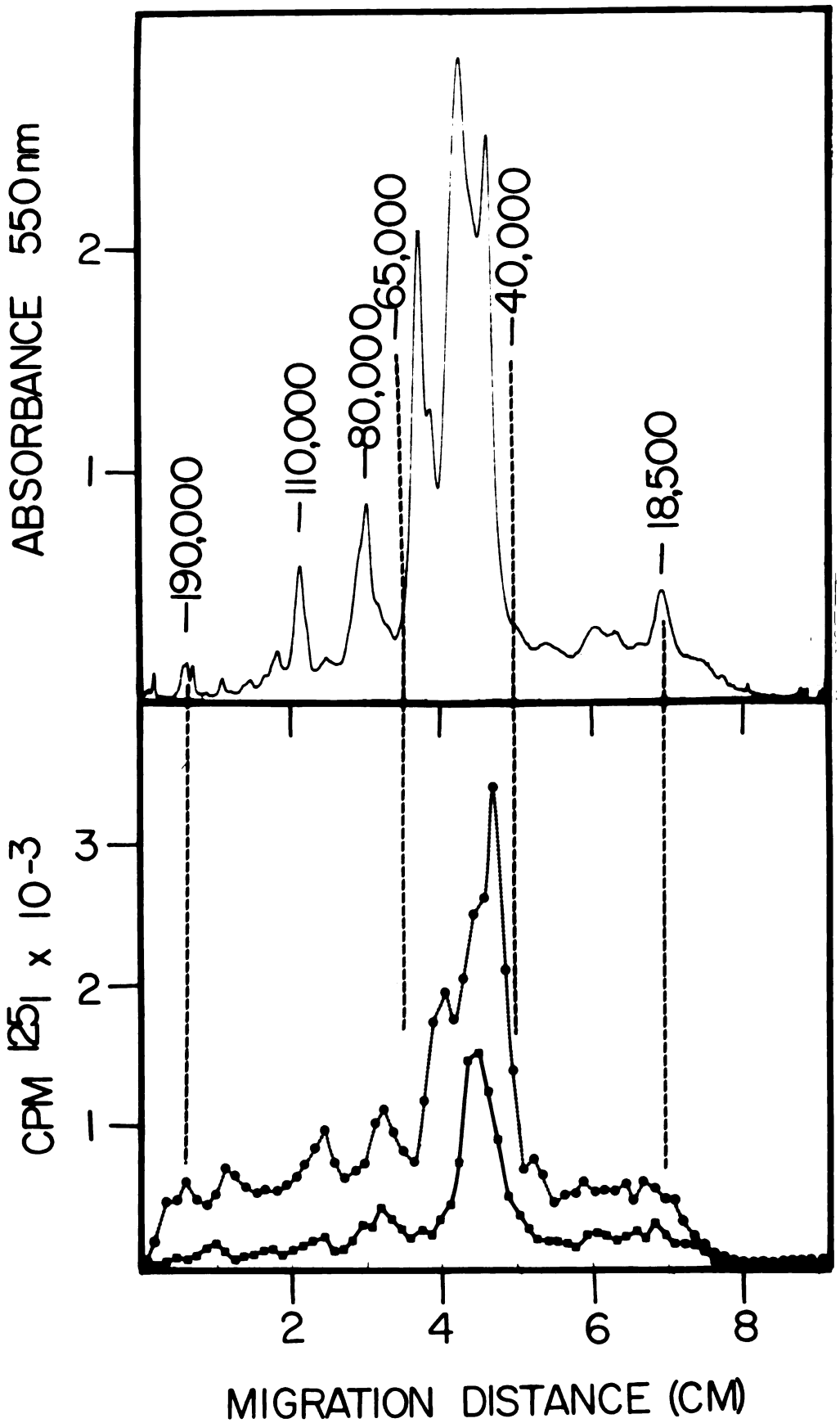

ABSORBANCE 550nm
2
1
190,000
110,000
80,000
65,000
40,000
18,500
CPM 125I x 10-3
3
2
1
2
4
6
8
MIGRATION DISTANCE (CM)

Figure 8

Trypsin Treatment of ^{125}I-Labeled Microsomes

To further demonstrate that lactoperoxidase was labeling exterior proteins, ^{125}I-labeled microsomes were incubated with trypsin. Trypsin cannot permeate the microsomal membranes but will cleave peptides from the outside of the membrane (29,31,32). Thus this protease should be capable of altering the ^{125}I-labeling pattern of microsomes if lactoperoxidase is indeed labeling exterior proteins. A time course for the solubilization of microsomal protein by trypsin (in the presence of 0.005% BHT) demonstrated that at 25°C, proteolysis was complete after 60 minutes (Figure 9). As can be seen from Figure 10, trypsin treatment also removed ^{125}I from microsomal proteins indicating that many of the proteins iodinated by lactoperoxidase are also substrates for this protease. Thus by this criterion too, lactoperoxidase was labeling exterior proteins.

To emphasize again the importance of carrying out membrane iodination in the presence of BHT, Figure 10C demonstrates the gel protein profile obtained from microsomes iodinated in the absence of BHT and then treated with trypsin. It can be seen that if the iodination is not carried out in the presence of BHT, lipid peroxidation causes an extensive breakdown in the structure of the membrane as demonstrated by the increased susceptibility of most membrane proteins to trypsin digestion.

A Comparison of the Iodination Profiles of Rough and Smooth Microsomes

Since completing these experiments on the structure of the rat liver endoplasmic reticulum, Kreibich *et al.* (131) have published the

Figure 9. PROTEIN SOLUBILIZED FROM RAT LIVER MICROSOMES BY TRYPSIN TREATMENT AT 25°C FOR VARYING PERIODS OF TIME

Sucrose containing sodium pyrophosphate-washed microsomes isolated from a PB-pretreated rat were suspended to a protein concentration of 6 mg/ml in 0.05 M Tris-HCl, pH 7.5 (at 25°C) containing 1 mM EDTA and 0.005% BHT. Trypsin was added in a ratio of 10 µg trypsin per mg of microsomal protein to samples of this suspension which were then incubated in parallel under N_2 at 25°C. The proteolytic reaction was terminated in these samples at varying lengths of time by adding soybean trypsin inhibitor at a ratio of 5 µg of trypsin inhibitor per µg of trypsin. The samples were centrifuged at 105,000 xg for 90 minutes and the supernatants were then assayed for protein released from the microsomes. (In the absence of the protease less than 5% of the microsomal protein was released after 90 minutes at 25°C.)

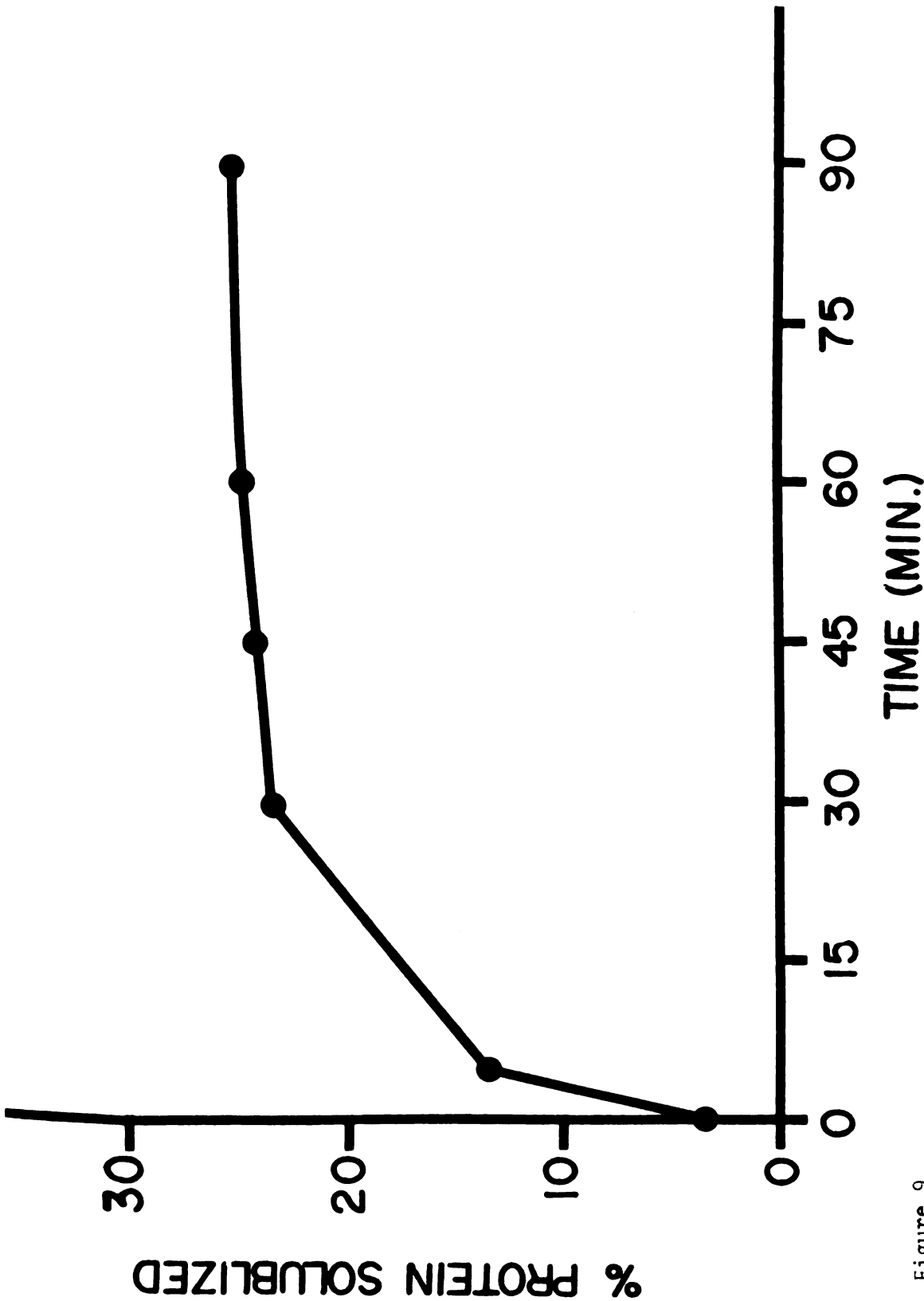


Figure 9

Figure 10. THE EFFECT OF TRYPSIN TREATMENT ON THE SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROTEIN AND ^{125}I-INCORPORATION PROFILES OF THE LIVER MICROSOMAL FRACTION FROM PB-PRETREATED RATS

Microsomal membranes were washed with sucrose containing sodium pyrophosphate and iodinated, as described in the Materials and Methods. The iodination mixture contained 4 μC ^{125}I/ml reaction. The membranes were then resuspended to a protein concentration of 6 mg/ml in 0.05 M Tris-HCl, pH 7.5 (at 25°C) containing 1 mM EDTA and 0.005% BHT for treatment as described below. After incubation under N_2 at 25°C for 60 minutes, the membranes were pelleted by centrifugation and suspended in 1% SDS-buffer for electrophoresis. The smooth lines represent the protein profiles obtained while the dotted lines designate the ^{125}I-incorporation profiles.

A. The electrophoresis profiles obtained from ^{125}I-labeled microsomes which were incubated in parallel with trypsin-treated microsomes; no protease was added to this sample, however. 55 μg of protein was applied to this gel.

B. The electrophoresis profiles obtained after ^{125}I-labeled microsomes were incubated with trypsin (10 μg/ml microsomal protein). 52 μg of protein was applied to the gel.

C. The electrophoresis profiles obtained when microsomes which were iodinated in the absence of BHT were then subject to trypsin treatment (10 μg/mg microsomal protein) in the presence of BHT. 40 μg of protein was applied to the gel.

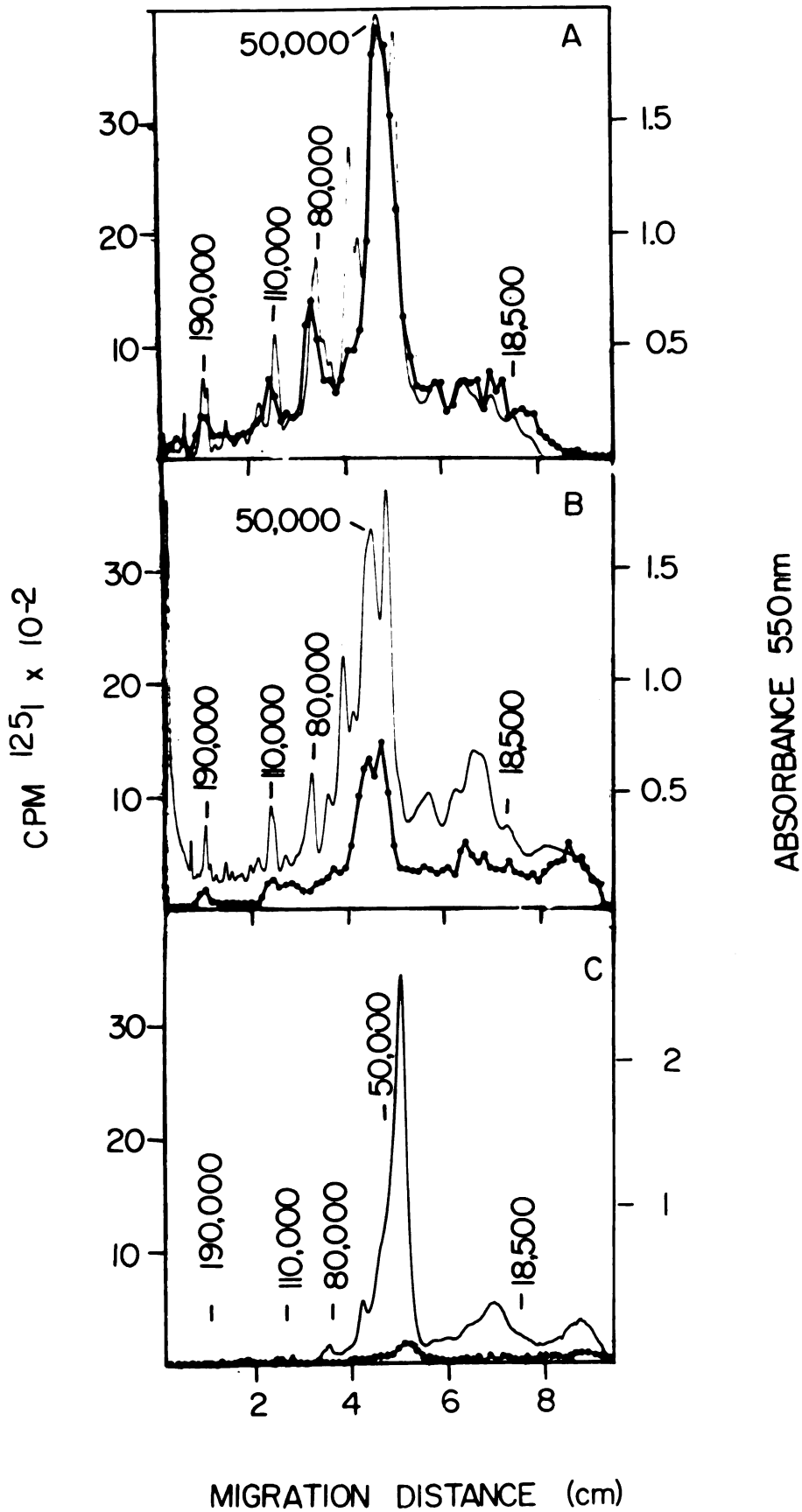

A
B
C
50,000
190,000
110,000
80,000
18,500
50,000
190,000
110,000
80,000
18,500
50,000
190,000
110,000
80,000
18,500
30
20
10
1.5
1.0
0.5
2
1
2
4
6
8
CPM 125I x 10-2
ABSORBANCE 550nm
MIGRATION DISTANCE (cm)

Figure 10

Figure 10. THE EFFECT OF TRYPSIN TREATMENT ON THE SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROTEIN AND ^{125}I-INCORPORATION PROFILES OF THE LIVER MICROSOMAL FRACTION FROM PB-PRETREATED RATS

Microsomal membranes were washed with sucrose containing sodium pyrophosphate and iodinated, as described in the Materials and Methods. The iodination mixture contained 4 μC ^{125}I/ml reaction. The membranes were then resuspended to a protein concentration of 6 mg/ml in 0.05 M Tris-HCl, pH 7.5 (at 25°C) containing 1 mM EDTA and 0.005% BHT for treatment as described below. After incubation under N_2 at 25°C for 60 minutes, the membranes were pelleted by centrifugation and suspended in 1% SDS-buffer for electrophoresis. The smooth lines represent the protein profiles obtained while the dotted lines designate the ^{125}I-incorporation profiles.

A. The electrophoresis profiles obtained from ^{125}I-labeled microsomes which were incubated in parallel with trypsin-treated microsomes; no protease was added to this sample, however. 55 μg of protein was applied to this gel.

B. The electrophoresis profiles obtained after ^{125}I-labeled microsomes were incubated with trypsin (10 μg/ml microsomal protein). 52 μg of protein was applied to the gel.

C. The electrophoresis profiles obtained when microsomes which were iodinated in the absence of BHT were then subject to trypsin treatment (10 μg/mg microsomal protein) in the presence of BHT. 40 μg of protein was applied to the gel.

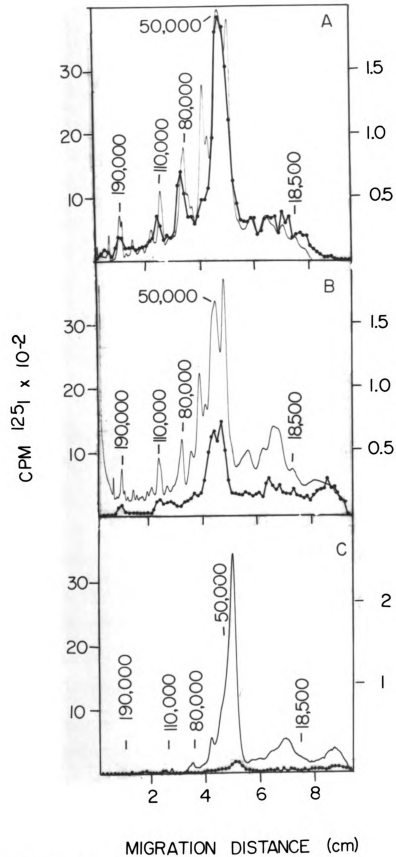

A
B
C
50,000
190,000
110,000
80,000
18,500
CPM 125I x 10-2
ABSORBANCE 550nm
MIGRATION DISTANCE (cm)

Figure 10

results of similar studies in which lactoperoxidase-catalyzed iodination was used to examine the structure of the rough endoplasmic reticulum. They found a similar iodination pattern to that presented in Figure 5, however the major peak of radioactivity was associated with proteins of less than 30,000 daltons. This peak they ascribed to ribosomal proteins. Since in the studies reported in this chapter the membranes were washed free of ribosomes before ^{125}I-labeling, such a peak of ^{125}I would not be expected in the gels. Indeed it was found that the ^{125}I-labeling patterns of rough and smooth microsomes were identical if the membranes were first washed with 0.3 M sucrose containing 0.1 M sodium pyrophosphate pH 7.5 (Figure 11).

Discussion

In this chapter I have presented the results of studies which were conducted to examine the feasibility of using enzymatic iodination to investigate the topography of membrane proteins in the rat liver endoplasmic reticulum. It was shown that this technique can be used with microsomes if care is taken to avoid the peroxidation of membrane lipids during the iodination. Inclusion of the antioxidant, BHT, in the iodination mixture completely inhibits the peroxidation of membrane lipids and increases the incorporation of ^{125}I into microsomes by 2-fold. In addition, inclusion of this antioxidant also preserves cytochrome P_{450} and aminopyrine demethylase activity during the iodination, probably as a result of its ability to inhibit lipid peroxidation. If BHT is used, the general structure of the membrane also appears to be preserved as evidenced by the difference in the

Figure 11. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROTEIN AND ^{125}I-INCORPORATION PROFILES OF ROUGH AND SMOOTH LIVER MICROSOMAL FRACTIONS FROM A CONTROL RAT

Rough and smooth microsomal membranes were washed with sucrose containing sodium pyrophosphate, iodinated, and prepared for electrophoresis, as described in the Materials and Methods. The iodination mixture contained 2 μC ^{125}I/ml reaction. 50 μg of protein was applied to each gel. The smooth line represents the protein profile while the dotted line designates the ^{125}I-incorporation pattern.

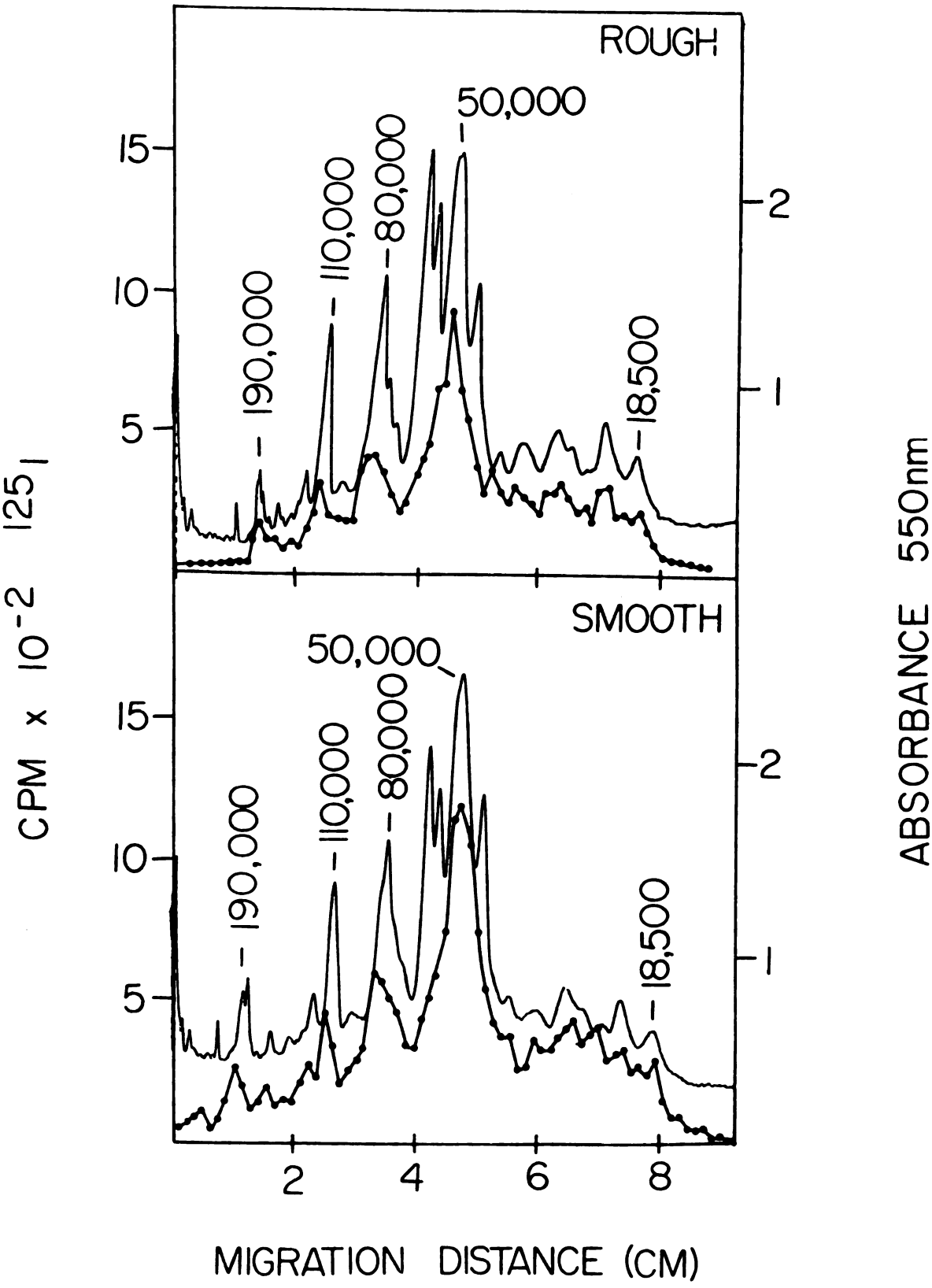

ROUGH
50,000
80,000
110,000
190,000
18,500
SMOOTH
50,000
80,000
110,000
190,000
18,500
15
10
5
2
4
6
8
2
1
CPM x 10-2 125I
ABSORBANCE 550nm
MIGRATION DISTANCE (CM)

Figure 11

susceptibility of membrane proteins to tryptic digest after iodination in the presence or absence of the antioxidant.

If care is taken to prevent lipid peroxidation, the enzymatic-iodination technique appears to be an effective probe for specifically labeling proteins exposed to the exterior of the microsome. This conclusion is based primarily upon two observations. First of all, iodination of microsomal proteins is much more specific before lipids are extracted from the membrane than after. This demonstrates that while most of the proteins are capable of being iodinated, when they are incorporated into the membrane structure, they are not all equally available to react with lactoperoxidase. It is reasonable to suspect that this is because lactoperoxidase cannot diffuse through the membrane but can only act at its surface. Secondly, tryptic digest removes most of the ^{125}I incorporated into microsomal proteins from the membrane. This protease has previously been shown to cleave peptides only from the exterior surface of the microsome (29,31,32). Thus the ability of this protease to remove ^{125}I-labeled peptides suggests that iodination must also only occur on the exterior surface.

It therefore appears that since lactoperoxidase only iodinates exterior proteins, several general conclusions on the arrangement of proteins in the endoplasmic reticulum can be drawn from the experiments presented in this chapter. First of all, on the basis of SDS-polyacrylamide gel electrophoretic analysis, it appears that rough and smooth microsomal membranes have similar protein compositions when ribosomes and adsorbed proteins are removed. Since the ^{125}I-labeling patterns obtained for the protein constituents of these membranes are similar after lactoperoxidase-catalyzed protein iodination, it also can

be concluded that the spatial arrangement of proteins in these membranes is alike. Other investigators have concluded that these membranes have similar lipid compositions (9) and enzymatic components (10,11,12). Thus, overall, it appears that the membranes of the rough and smooth compartments of the endoplasmic reticulum are similarly constructed. These membranes are composed of a few major protein components and a large array of minor components. In this respect they are similar to many other cellular membranes (132). In microsomes the major components have apparent molecular weights ranging from 40,000 to 60,000 daltons. The size of the minor components, however, span the range from below 15,000 to above 200,000. Many of the minor constituents are located on the external surface of the membrane since they are easily ^{125}I-labeled. Only major constituents having molecular weights of 50,000 are also located on this surface, however. Since isolated microsomes are closed vesicles which have retained the normal cellular orientation of the endoplasmic reticulum, one should remember that the exterior of a microsome is actually the side of the endoplasmic reticulum membrane which is in contact with the cytoplasm of the cell. It therefore appears that in the cell, many of the minor components of the endoplasmic reticulum are in direct contact with the cytoplasm but that many of the major protein components are either buried within the membrane or oriented on its luminal face. It must be remembered, however, that such conclusions are always made on the assumption that all of the proteins located on the exterior of the membrane can be iodinated by lactoperoxidase.

It has been theorized by many scientists that there is a continuity of membranes within the organelles of cells. On the basis of

both electron microscopic and biochemical criteria it has been suggested that membranes of other cellular organelles may be derived from the endoplasmic reticulum. The presence of components of the microsomal electron transport chains in the outer nuclear membrane (133-136). peroxisomes (137), and the outer mitochondrial membranes (138) of liver cell organelles has been cited as evidence for this notion. Furthermore, these membranes have lipid compositions similar to that of the endoplasmic reticulum (137,139,140). Also, morphological observations have been made of a direct continuity between the endoplasmic reticulum and peroxisomal (2) and the outer nuclear membranes (5). While some similarities in the protein composition of outer mitochondrial membranes (115), nuclear membranes (141), and endoplasmic reticulum have already been observed by SDS-polyacrylamide gel electrophoresis, very little is known about the protein composition of peroxisomal membranes. It will be interesting to see if these membranes all have similar protein compositions and if the spatial arrangement of proteins within these membranes is also similar to that described in this chapter for the endoplasmic reticulum. It is hoped that in the future this type of study will be undertaken to enhance our knowledge of membrane continuity within cells.

In the chapters which follow I have used the techniques of enzymatic iodination and SDS-polyacrylamide gel electrophoresis to investigate specific questions about two protein components of the microsomal mixed-function oxidase system. These components are NADPH-cytochrome c reductase and cytochrome P_{450}, and answers will be sought to questions such as: What is the molecular weight of the native NADPH-cytochrome c reductase? Do multiple forms of this reductase

exist? What is the position of cytochrome P_{450} in the microsomal membrane? And, do multiple cytochrome P_{450} hemoproteins exist in microsomes?

CHAPTER TWO

MULTIPLICITY OF NADPH-CYTOCHROME C REDUCTASE ENZYMES IN RAT LIVER MICROSOMES

Abstract

As one criteria for determining if multiple forms of NADPH-cytochrome c reductase are present in rat liver microsomes, the molecular weights of the "native" reductases from control, PB-, and 3-MC-pretreated rats were compared by SDS-polyacrylamide gel electrophoresis. This comparison was performed using a method which was developed to determine the molecular weight of the "native" reductase present in microsomes from PB-pretreated rats. This method involved immunoprecipitation of the enzyme from sodium deoxycholate-solubilized, ^{125}I-labeled microsomal proteins. The immunoprecipitation was carried out using antibody prepared against a purified protease-solubilized fragment of the reductase. The immunoprecipitate was then subjected to SDS-polyacrylamide gel electrophoresis and the molecular weight of the reductase was determined by scanning the gel for ^{125}I. Using this technique, the molecular weight of the "native" reductases from the liver microsomes from control, PB-, and 3-MC-pretreated rats were all shown to be the same, 79,000. This comparison then suggests that the reductase enzymes present in all three types of microsomes are identical.

A comparison of the molecular weight of the "native" reductase (79,000) with that of the proteolytically solubilized fragment used for antibody production (71,000) suggests that the structure for the "native" enzyme may be similar to that of two other microsomal proteins, NADH-cytochrome b_5 reductase and cytochrome b_5. These proteins appear to be amphipathic, consisting of a single polypeptide chain containing a large hydrophilic segment which contains the active site of the enzyme and is exposed to the cytoplasm of the cell and a smaller hydrophobic "tail" segment which interacts with the phospholipids of the membrane. Proteases cleave the hydrophilic portion from the membrane, leaving behind the hydrophobic "tail" segment. If this is also the case with NADPH-cytochrome c reductase, this enzyme has a "tail" segment of approximately 70 amino acid residues.

Introduction

Numerous investigators have attempted to determine if different NADPH-cytochrome c reductase enzymes are present in the liver microsomes from control, PB- and 3-MC pretreated rats. The existence of different forms of this enzyme would suggest that the different mixed-function oxidase activities observed in these microsomes are due to the presence of different electron transport chains in the microsomes. Kuriyama, et al. (58) have shown that the reductase enzymes from the liver microsomes of both normal and PB-induced rats can be solubilized by trypsin and behave similarly during chromatography on Sephadex G-100 and on DEAE-cellulose. In addition, these trypsin-solubilized enzymes appear immunologically identical on the basis of Ouchterlony double diffusion analysis against antibody prepared to a purified form of the

trypsin-solubilized enzyme from PB-microsomes. Because these studies were made using trypsin-solubilized enzymes, however, they cannot be considered conclusive proof that the "native" enzymes present in the microsomes from control and PB-pretreated rats are the same. Trypsin treatment undoubtedly modifies this protein as suggested by the fact that the trypsin-solubilized enzyme cannot replace the partially purified reductase fraction in a reconstituted microsomal hydroxylation system (142,143).

Antibody prepared to the trypsin-solubilized enzyme from the liver microsomes of PB-pretreated rats has also been shown to inhibit, to the same extent, the NADPH-cytochrome c reductase activity present in the microsomes from normal and PB-pretreated rats (53,58) and in the microsomes from 3-MC-pretreated rats (53). While this property suggests that the reductase found in all three types of microsomes are immunologically very similar, it too cannot be taken as conclusive proof that they are the same. Similar antibodies, also prepared to liver microsomal NADPH-cytochrome c reductase, appear capable of inhibiting the reductase from adrenal microsomes, and the enzymes from liver and adrenal microsomes are not immunologically identical on the basis of Ouchterlony double diffusion analysis (55).

More recently, Lu et al. (73) have attempted to study the possible existence of multiple forms of the reductase by using partially purified detergent-solubilized NADPH-cytochrome c reductase fractions from PB- and 3-MC-microsomes to reconstitute hydroxylation activity with a partially purified preparation of cytochrome P_{450} from PB-pretreated rats. These studies were undertaken to determine if the different reductases played a role in conferring different

substrate specificites on the reconstituted system. They concluded that for some substrates the reductases from either type of microsome were equally effective in this system while for others the particular reductase did appear to influence substrate specificity. These results then suggested that different forms of the "native" reductases may exist in the microsomes from 3-MC and PB-pretreated rats. Because no one has been able to isolate these proteins after detergent-solubilization, however, it has been impossible to compare any of the physical properties of the "native" reductases present in the different microsomes.

In this chapter, a method is described by which the molecular weights of the "native" forms of NADPH-cytochrome c reductase present in the liver microsomes isolated from control, PB-, and 3-MC-pretreated rats could be compared without the prior isolation of the enzymes. This method, first developed to determine the molecular weight of the "native" reductase from the liver microsomes of a PB-pretreated rat, involved: (1) the lactoperoxidase-catalyzed radio-iodination of the "native" reductase in microsomes, (2) solubilization of these microsomes in sodium deoxycholate, (3) immunoprecipitation of the ^{125}I-labeled reductase from the detergent-solubilized microsomes using an antibody prepared to a proteolytically-solubilized form of the reductase from PB-microsomes, (4) solubilization of the immunoprecipitate in SDS for polyacrylamide gel electrophoresis, and (5) identification of the "native" reductase by gel fractionation and ^{125}I-gamma counting. Similar techniques have previously been used to study immunoglobulins (144), O-antigens (145), and H-2 alloantigens (146,147) from cell surfaces.

Because the antibody to the proteolytically solubilized reductase from PB-microsomes would cross-ract with the reductases in control and 3-MC-microsomes, this immunoprecipitation technique could then be used to compare the molecular weights of the reductases from the liver microsomes of control, PB-, and 3-MC-pretreated rats.

Materials and Methods

Materials

Male Sprague-Dawley rats weighing between 75-100 g were obtained from Spartan Research Animals, Inc., Haslett, Michigan.

3-Methylcholanthrene (3-MC) was obtained from the Sigma Chemical Company, St. Louis, Missouri. Sodium deoxycholate was obtained from the Schwartz-Mann Division of Becton Dickinson and Company, Orangeburg, New York. Bromelain was a gift from the Dole Company, San Jose, California. Potassium ferricyanide was obtained from Mallinckrodt, St. Louis, Missouri. The agar used for Ouchterlony double diffusion analysis was special Nobel Grade obtained from Difco Laboratories, Detroit, Michigan.

The sources of other reagents have been listed in chapter one.

Drug Pretreatment of Animals

Rats were pretreated with either PB (50 mg/kg in saline) by daily i.p. injection 5 days prior to sacrifice or with 3-MC (20 mg/kg in corn oil) by i.p. injection 36 and 24 hours prior to sacrifice. The liver microsomes isolated from PB-pretreated rats are referred to as PB-microsomes while those isolated from rats pretreated with 3-MC are termed 3-MC-microsomes.

Preparation of Bromelain-Solubilized NADPH-Cytochrome c Reductase from the Liver Microsomes of PB-Pretreated Rats

Bromelain-solubilized NADPH-cytochrome c reductase was prepared by Dr. Thomas Pederson and the purification procedure used has been extensively described in his thesis (98). This enzyme is referred to as the bromelain reductase.

Preparation of Antibody to Bromelain-Solubilized NADPH-Cytochrome c Reductase

Antibody was prepared to the bromelain reductase by Mr. John Buege. The methods used for preparation of the antibody and purification of IgG from antiserum have been described by Pederson (98). The IgG fraction, purified from either antiserum or pre-immune serum, was used in these studies.

Ouchterlony Double Diffusion Analysis

Ouchterlony double diffusion analysis (148) was performed in disposable plastic petri dishes (100 x 15 mm) containing 1% agar dissolved in 0.015 M sodium phosphate, pH 8.0, 0.1% sodium deoxycholate, and 0.02% sodium azide. To prepare a dish, a stock agar mix was melted by heating in a boiling water bath and 13 ml of the agar was pipetted into a petri dish. After the agar had cooled and solidified, a punch was used to put the well pattern into the agar. Agar was removed from the wells using an aspirator. Antigen was added to the outer wells and antibody to the center well. The plates were developed at room temperature for 24 hours.

Enzymatic Assays

NADPH-dependent cytochrome c reduction was assayed as described by Pederson (98). NADPH-dependent ferricyanide reduction was assayed at 25°C in a reaction mixture containing 0.5 mM potassium ferricyanide and 0.1 mM NADPH in 0.3 M phosphate buffer, pH 7.5. The reduction of ferricyanide was measured at 420 nm in a Perkin-Elmer model 124 spectrophotometer. The rate is expressed as micromoles of ferricyanide reduced per minute using a millimolar extinction coefficient of 1.02 (149).

Lactoperoxidase-Catalyzed Iodination of Microsomal Membranes and 1% SDS-Polyacrylamide Gel Electrophoresis

Both procedures were performed as described in chapter one. The iodination mixture contained 10 μC $^{125}I^-$/ml.

Immunoprecipitation of "Native" NADPH-Cytochrome c Reductase from Detergent-Solubilized Rat Liver Microsomal Proteins

For experiments in which the reductase was immunoprecipitated from ^{125}I-labeled PB-microsomal protein, 2.0 mg of ^{125}I-labeled microsomal membranes (4,000,000 cpm/mg) were mixed with 7 mg of unlabeled microsomes and suspended to a concentration of 4 mg/ml in 0.05 M Tris-HCl (pH 7.5 at 25°C) containing 1.5% sodium deoxycholate and 10 mM EDTA. A small amount of material, not solubilized by the detergent, was removed by centrifugation at 130,000 x g for 90 minutes. IgG prepared from immune serum was added to the supernatant in a ratio of 1 mg IgG to 1 mg of microsomal protein and incubated at 0-4°C for 12 hours. The final concentration of sodium deoxycholate in this

mixture was 0.75%. A control using pre-immune IgG was run in parallel but no immunoprecipitate formed. The precipitate resulting from the antibody was centrifuged at 3,000 x g for 10 minutes and washed 3 times with 0.05 M Tris-HCl (pH 7.5 at 25°C) containing 1% sodium deoxycholate and 10 mM EDTA. The immunoprecipitate was then either suspended in 0.05 M Tris-HCl (pH 7.5 at 25°C) and 10 mM EDTA for enzymatic assay or dissolved in 1% SDS containing 7% sucrose, 10 mM Tris-HCl (pH 8.0 at 25°C), 1 mM EDTA, and 40 mM dithiothreitol before heating at 100°C for 15 minutes. After cooling, 10 μg/ml pyronin B tracking dye was added and the samples were applied to polyacrylamide gels for electrophoresis in 1% SDS. After electrophoresis the gels were either stained with Coomassie blue and scanned for protein at 550 nm using a Gilford spectrophotometer or immediately fractionated using a Savant Autogel Divider for gamma counting to determine the ^{125}I distribution.

Experiments to immunoprecipitate the "native" reductase from unlabeled liver microsomes from control, PB-, and 3-MC-pretreated rats were performed similarly except that IgG was added in a ratio of 2 mg IgG to 1 mg of microsomal protein because of the lower titre of antibody in the IgG used for this experiment.

Results

Ouchterlony Double Diffusion Analysis of the Precipitin Reactions Between the Antibody to Bromelain Reductase, the Bromelain Reductase, and Detergent-Solubilized Liver Microsomal Proteins from a PB-Pretreated Rat

The feasibility of using antibody to the bromelain reductase to precipitate the "native" reductase from detergent-solubilized

PB-microsomes was initially investigated by Ouchterlony double diffusion analysis. As can be seen in Figure 12, this experiment demonstrated that the antibody would precipitate a single component from detergent-solubilized PB-microsomes. Since the precipitin line formed with the detergent-solubilized microsomal proteins fused with that formed with bromelain reductase, the microsomal component precipitated was antigenically similar to the bromelain reductase and most likely was the "native" reductase. IgG isolated from pre-immune serum would not form a precipitin line with any of the samples.

Enzymatic Characterization of the Immunoprecipitate Formed Between the Antibody and Detergent-Solubilized Microsomes

An excellent way to more clearly determine that the microsomal component precipitated by the antibody was the "native" reductase was to assay for the activity of this enzyme in an immunoprecipitate. When antibody was added to the solubilized microsomes to form an immunoprecipitate, the ability of the detergent-solubilized enzyme to reduce cytochrome c and ferricyanide was inhibited 97% and 66%, respectively (Table VII). Antibody to a proteolytically solubilized form of the reductase has previously been shown to inhibit reactions catalyzed by this enzyme (53,55,57,58,98). Since all the residual activity of the enzyme was found in the immunoprecipitate, however, it appeared as if the microsomal component immunoprecipitated by the antibody was indeed the "native" reductase.

Figure 12. OUCHTERLONY DOUBLE DIFFUSION ANALYSIS OF THE PRECIPITIN REACTIONS BETWEEN ANTIBODY TO BROMELAIN-SOLUBILIZED NADPH-CYTOCHROME C REDUCTASE, THE BROMELAIN REDUCTASE, AND DETERGENT-SOLUBILIZED LIVER MICROSOMAL PROTEINS FROM A PB-PRETREATED RAT

Ouchterlony double diffusion analysis was performed as described in the Materials and Methods. The center well contains anti-NADPH-cytochrome c reductase IgG (1.62 mg of protein). The outer wells contain various antigens and are numbered clockwise from the top (No. 1). Wells 1 and 3 contain 0.2 and 0.4 mg of microsomal protein, respectively (5 mg/ml in 2% sodium deoxycholate). Wells 2 and 4 contains 3 and 10 μg of purified, bromelain-solubilized NADPH-cytochrome c reductase, respectively. Well 5 contains buffer and well 6 contains 2% sodium deoxycholate.

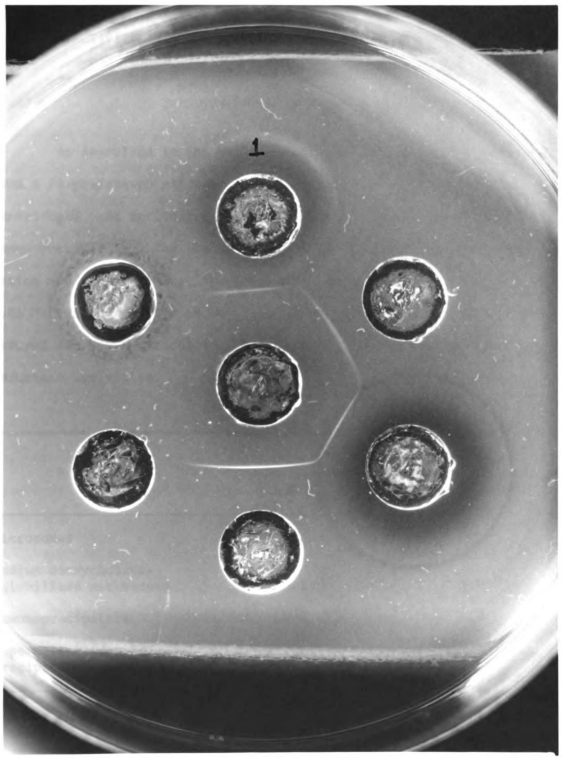


Figure 12

TABLE VII

NADPH-CYTOCHROME C AND NADPH-FERRICYANIDE REDUCTASE ACTIVITIES IN THE IMMUNOPRECIPITATE FORMED BETWEEN ANTIBODY TO BROMELAIN REDUCTASE AND DETERGENT-SOLUBILIZED LIVER MICROSOMAL PROTEIN FROM A PB-PRETREATED RAT

As described in the Material and Methods, liver microsomes from a PB-pretreated rat were solubilized in sodium deoxycholate, centrifuged, and antibody was added to the supernatant to form an immunoprecipitate. The immunoprecipitate was collected by centrifugation and after washing, the immunoprecipitate was suspended in 0.05 M Tris-HCl (pH 7.5 at 25°C) and 10 mM EDTA. All fractions were assayed for NADPH-cytochrome c reductase and NADPH-ferricyanide reductase activities.

Fraction	μmoles of receptor reduced/minute	
	Cytochrome c	Ferricyanide
Microsomes	2.1	3.1
Sodium deoxycholate-solubilized microsomes	2.1	3.0
Immunoprecipitate	0.1	1.0
Supernatant from immuno-precipitate	0.0	0.0

SDS-Polyacrylamide Gel Electrophoresis of the Protein Components of the Immunoprecipitate Form Between the Antibody to Bromelain Reductase and Detergent-Solubilized PB-Microsomes

The molecular weights of the protein components in the immunoprecipitate could be determined by solubilizing the immunoprecipitate in SDS and electrophoresing the solubilized proteins on SDS-polyacrylamide gels. Determination of which of the components was the "native" reductase, however, required that this protein be labeled with a specific probe which would differentiate it from the polypeptide components contributed by the antibody. Since the reductase has been shown by others to be located on the exterior of the microsome (29), this protein could be ^{125}I-labeled in the microsomes by the lactoperoxidase-catalyzed protein iodination technique described in chapter one. The ^{125}I-label could then be used as a specific marker for the reductase on SDS-gels run of the immunoprecipitate. In Figure 13, the results are shown of an experiment in which the immunoprecipitate formed between the antibody and ^{125}I-labeled detergent-solubilized liver microsomes isolated from a PB-pretreated rat was analyzed by SDS-gel electrophoresis. The protein and ^{125}I-labeling patterns of the immunoprecipitate are compared with those of the ^{125}I-labeled microsomal proteins from which the reductase was immunoprecipitated. The patterns for total microsomal proteins are typical of these normally obtained, as discussed in chapter one. The patterns for the immunoprecipitate are fairly simple and indicate that while the immunoprecipitate appears to contain three protein components, only one of the components is ^{125}I-labeled. The ^{125}I-labeled component (the "native" reductase) has a molecular weight of 79,000 $\pm$ 1,500 (average and standard deviation

Figure 13. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROTEIN AND ^{125}I-DISTRIBUTION PROFILES OF TOTAL PB-MICROSOMAL PROTEINS AND THE IMMUNOPRECIPITATE FORMED FROM SODIUM DEOXYCHOLATE-SOLUBILIZED PB-MICROSOMAL PROTEINS AND THE REDUCTASE ANTIBODY.

After sucrose containing sodium pyrophosphate-washing of microsomes, the membrane proteins were enzymatically iodinated. ^{125}I-labeled microsomal proteins were than suspended in sodium deoxycholate and immunoprecipitation was carried out as described in the Materials and Methods. (a) ^{125}I-Distribution obtained after enzymatic iodination of rat liver microsomal proteins; (b) protein scan of total microsomes; (c) ^{125}I-distribution in the immunoprecipitate; and (d) protein scan of the immunoprecipitate. Molecular weight markers, based on R_f values, were determined independently for each gel.

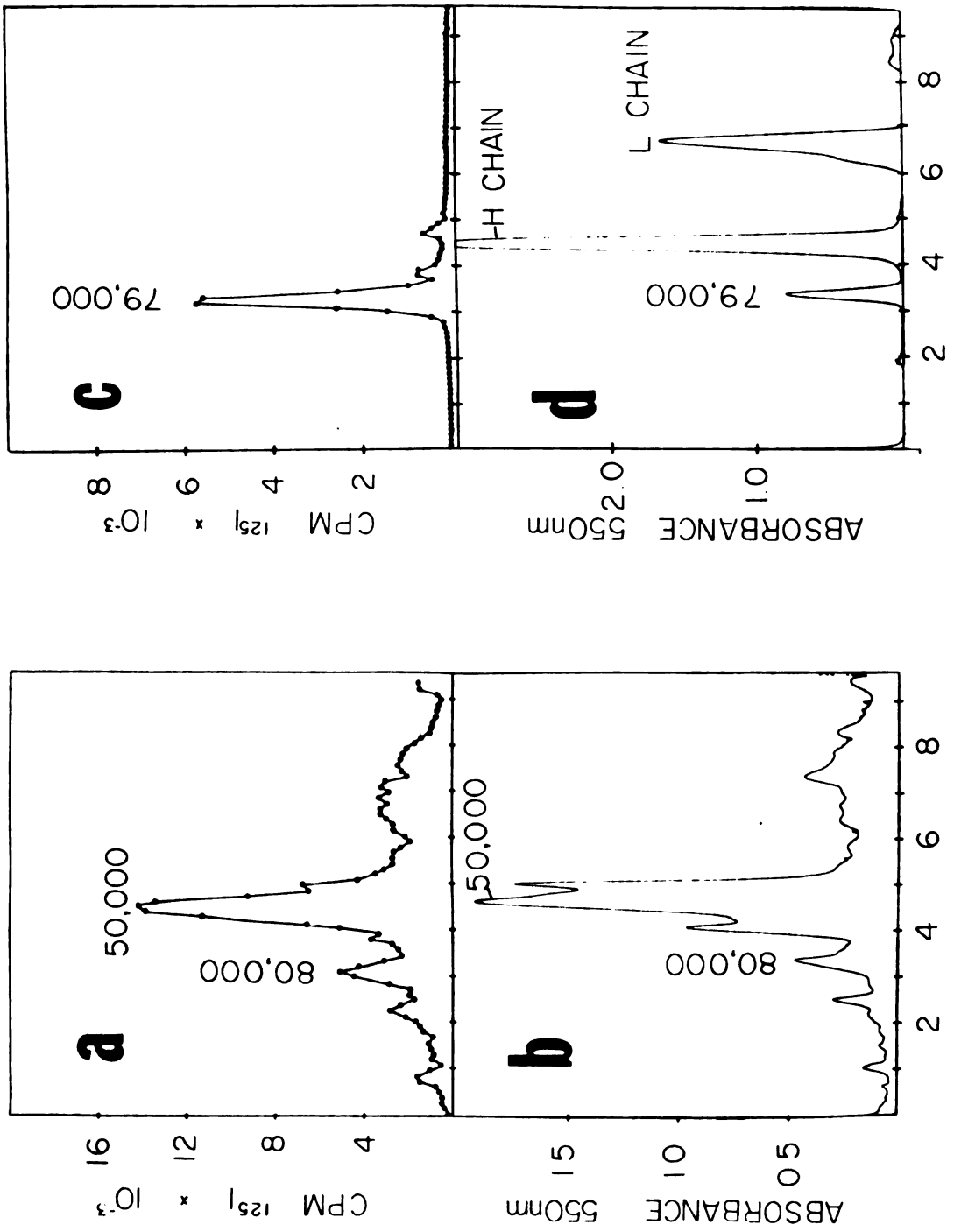

a
50,000
80,000
b
50,000
80,000
c
79,000
d
79,000
H CHAIN
L CHAIN
CPM 125I x 10-3
ABSORBANCE 550nm
MIGRATION DISTANCE CM

Figure 13

Figure 13. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROTEIN AND ^{125}I-DISTRIBUTION PROFILES OF TOTAL PB-MICROSOMAL PROTEINS AND THE IMMUNOPRECIPITATE FORMED FROM SODIUM DEOXYCHOLATE-SOLUBILIZED PB-MICROSOMAL PROTEINS AND THE REDUCTASE ANTIBODY.

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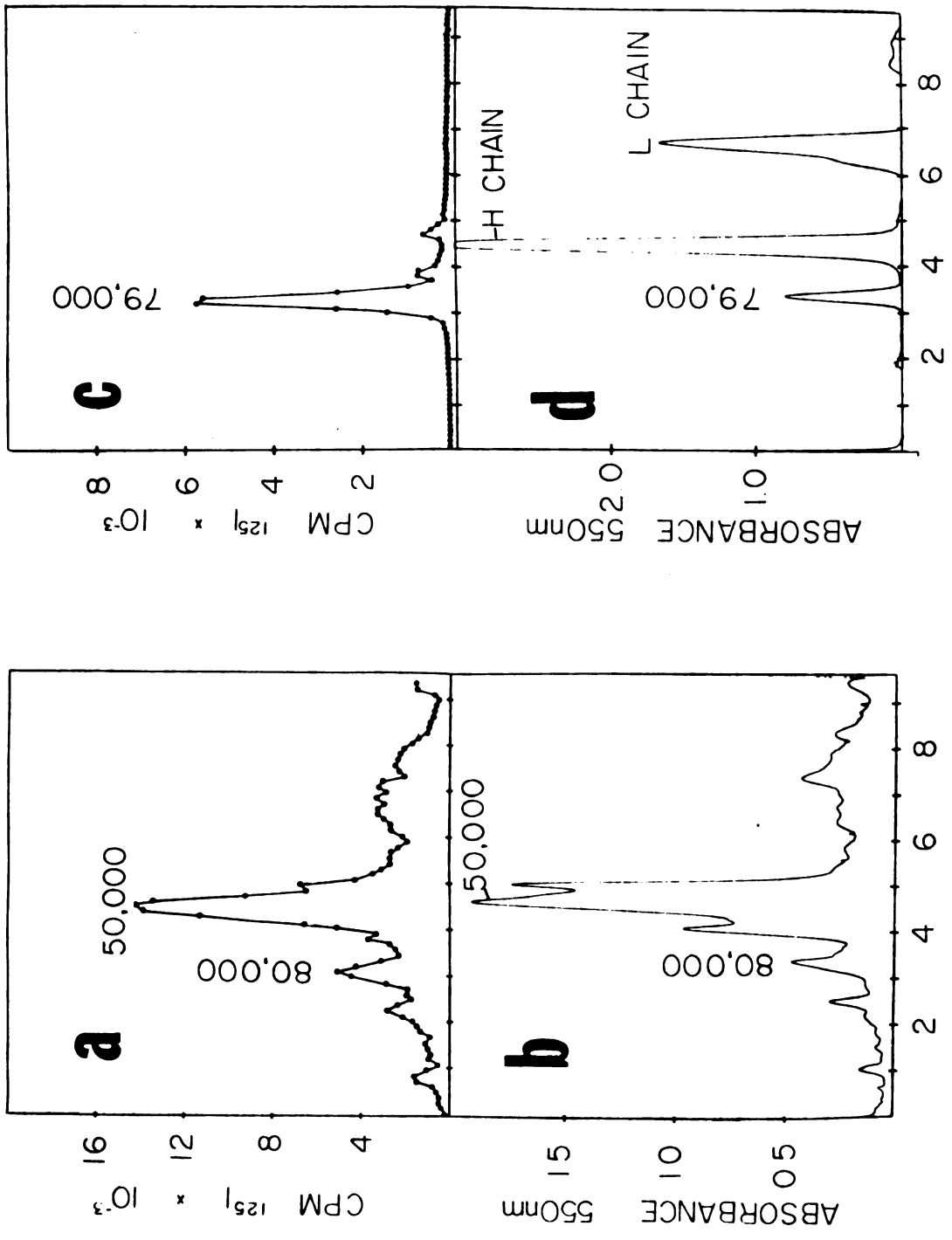
a
50,000
80,000
CPM ^{125}I × 10^{-3}
16
12
8
4
b
50,000
80,000
ABSORBANCE 550nm
1.5
1.0
0.5
2
4
6
8
c
79,000
CPM ^{125}I × 10^{-3}
8
6
4
2
H CHAIN
d
79,000
L CHAIN
ABSORBANCE 550nm
2.0
1.0
2
4
6
8
MIGRATION DISTANCE CM

Figure 13

of 5 determinations). Two unlabeled polypeptide chains are also present in the immunoprecipitate. These have molecular weights of 52,000 and 25,000, approximately the molecular weights of the heavy and light chains of IgG (150). In Figure 14, the SDS-gel protein pattern of the IgG used in these studies is compared to that of the immunoprecipitate. This confirms that the 52,000 and 25,000 molecular weight polypeptides present in the immunoprecipitate are components of the antibody.

A direct comparison of the protein profiles of the immunoprecipitate and total microsomal proteins (Figure 15) demonstrates that the "native" reductase is actually a very minor component of all the microsomal proteins. In fact it is not even well resolved in the total protein profile and appears to migrate with a group of 80,000 molecular weight proteins on the SDS-gels. This corresponds with previous data suggesting that the reductase actually represents less than 1% of the total microsomal protein (57-59,65). Also, looking at Figure 10 in chapter one, it can be seen that one of the most dramatic effects which trypsin treatment has on the microsomal membrane is the removal of proteins of approximately 80,000 molecular weight from the membrane. Since trypsin solubilizes nearly 100% of the reductase from the membrane (29), the loss of the reductase probably corresponds to the loss of the 80,000 molecular weight components observable on the SDS-gels after the microsomes have been treated with trypsin.

A Comparison of the Molecular Weights of "Native" Reductase and Bromelain Reductase

Figure 16 compares the Coomassie blue protein banding patterns for gels run on bromelain reductase, the immunoprecipitate formed

Figure 14. A COMPARISON OF THE SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROTEIN PROFILES OF IgG ISOLATED FROM ANTISERUM TO BROMELAIN REDUCTASE AND THE IMMUNOPRECIPITATE FORMED BETWEEN DETERGENT-SOLUBILIZED LIVER MICROSOMES FROM A PB-PRETREATED RAT AND THIS IgG

The upper scan is of the immunoprecipitate formed between the anti-reductase IgG and detergent-solubilized PB-microsomes while the lower scan is of the IgG itself. Samples were boiled in 1% SDS-buffer containing 40 mM dithiothreitol and electrophoresed, as described in the Materials and Methods.

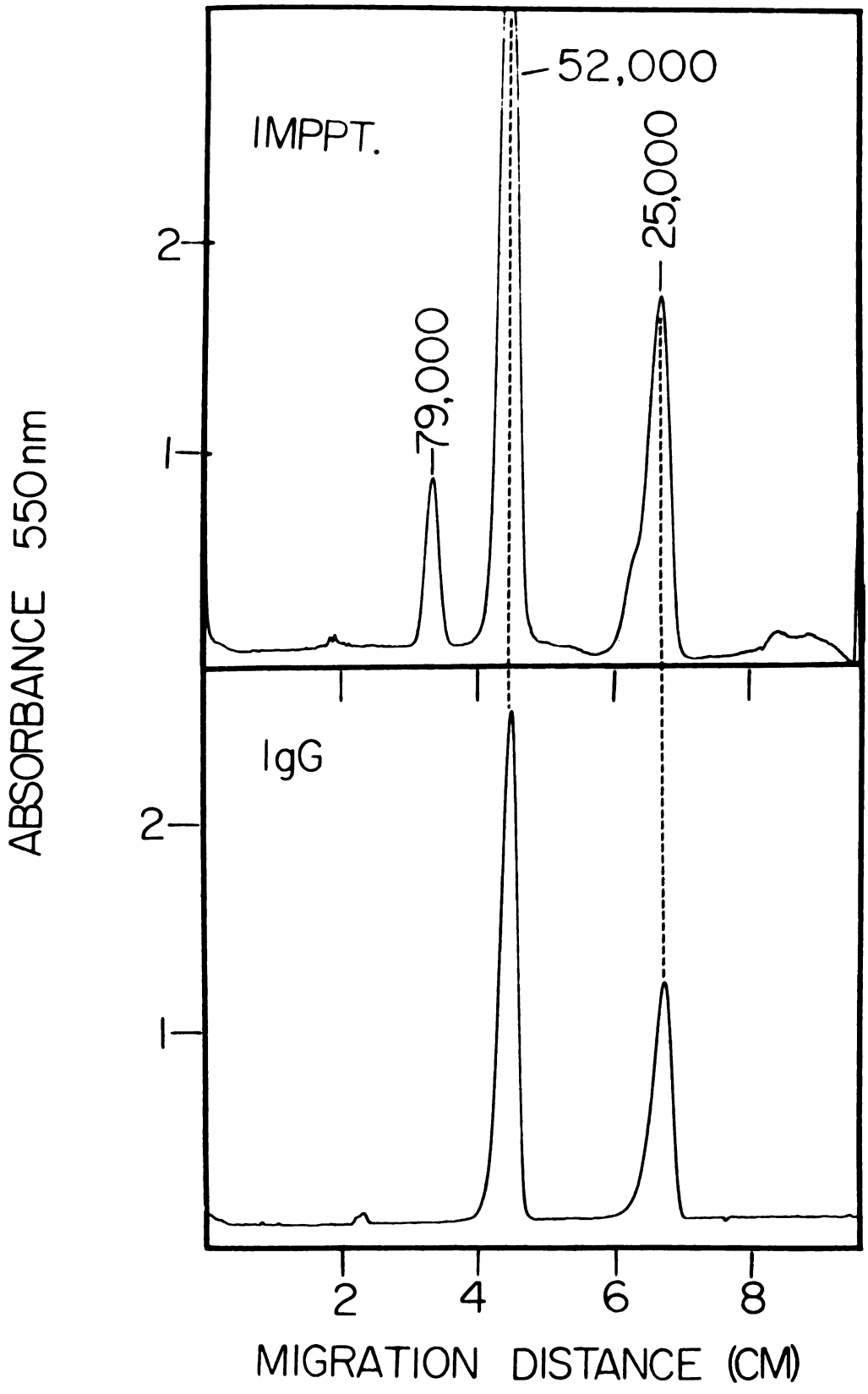
IMPPT.
52,000
25,000
79,000
2
1
IgG
2
1
ABSORBANCE 550nm
2
4
6
8
MIGRATION DISTANCE (CM)

Figure 14

Figure 14. A COMPARISON OF THE SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROTEIN PROFILES OF IgG ISOLATED FROM ANTISERUM TO BROMELAIN REDUCTASE AND THE IMMUNOPRECIPITATE FORMED BETWEEN DETERGENT-SOLUBILIZED LIVER MICROSOMES FROM A PB-PRETREATED RAT AND THIS IgG

The upper scan is of the immunoprecipitate formed between the anti-reductase IgG and detergent-solubilized PB-microsomes while the lower scan is of the IgG itself. Samples were boiled in 1% SDS-buffer containing 40 mM dithiothreitol and electrophoresed, as described in the Materials and Methods.

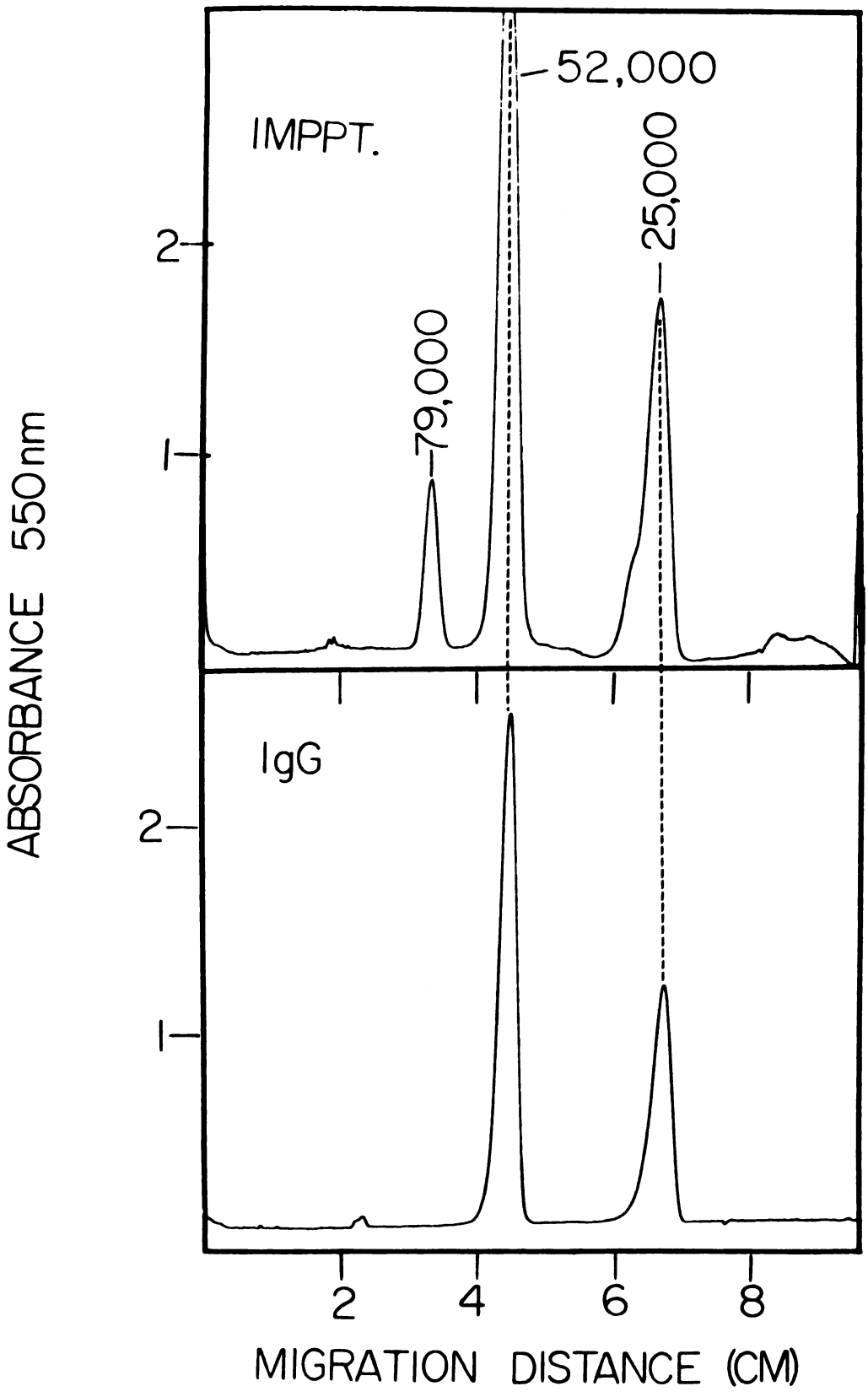
52,000
IMPPT.
25,000
79,000
2
1
ABSORBANCE 550nm
IgG
2
1
2
4
6
8
MIGRATION DISTANCE (CM)

Figure 14

Figure 15. A COMPARISON OF THE SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROTEIN PROFILES OF THE LIVER MICROSOMAL PROTEINS FROM A PB-PRETREATED RAT AND THE IMMUNOPRECIPITATE FORMED BETWEEN DETERGENT-SOLUBILIZED PB-MICROSOMAL PROTEINS AND ANTI-REDUCTASE IgG

The upper scan is of total microsomal proteins while the lower scan is of the immunoprecipitate formed between detergent-solubilized PB-microsomal proteins and antibody to Bromelain-solubilized NADPH-cytochrome c reductase.

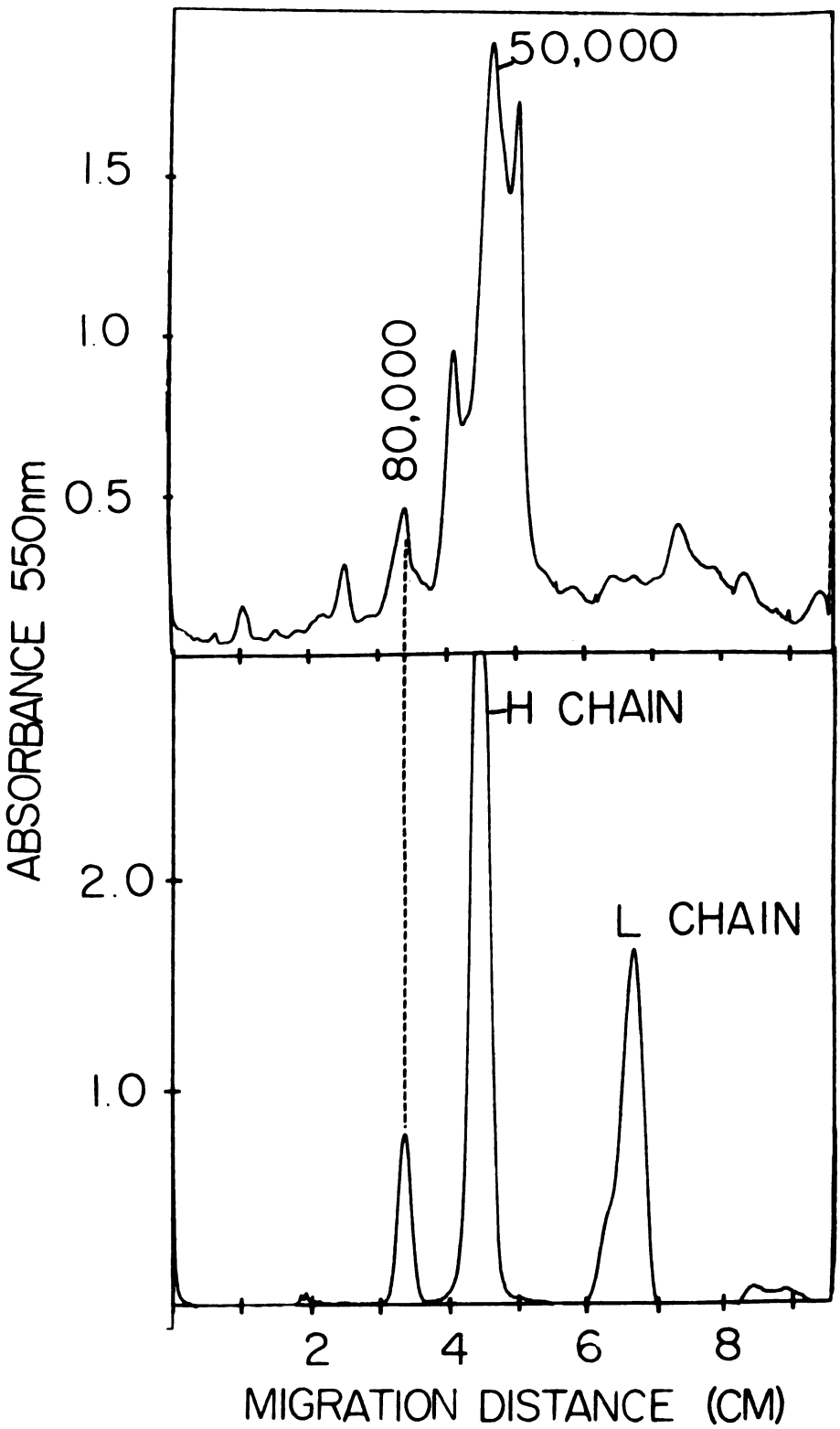
50,000
80,000
1.5
1.0
0.5
ABSORBANCE 550nm
H CHAIN
L CHAIN
2.0
1.0
2
4
6
8
MIGRATION DISTANCE (CM)

Figure 15

Figure 16. A COMPARISON OF THE SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROTEIN PROFILES OF PURIFIED BROMELAIN-SOLUBILIZED NADPH-CYTOCHROME C REDUCTASE AND THE IMMUNOPRECIPITATE FORMED BETWEEN DETERGENT-SOLUBILIZED MICROSOMAL PROTEINS FROM A PB-PRETREATED RAT AND ANTIBODY TO THE BROMELAIN REDUCTASE

Scan A is of the purified Bromelain-solubilized reductase, scan B is of the immunoprecipitate formed from detergent-solubilized microsomal proteins and the reductase antibody, and scan C is a mixture of the two samples. Molecular weight markers, based on R_f values were determined independently for each gel.

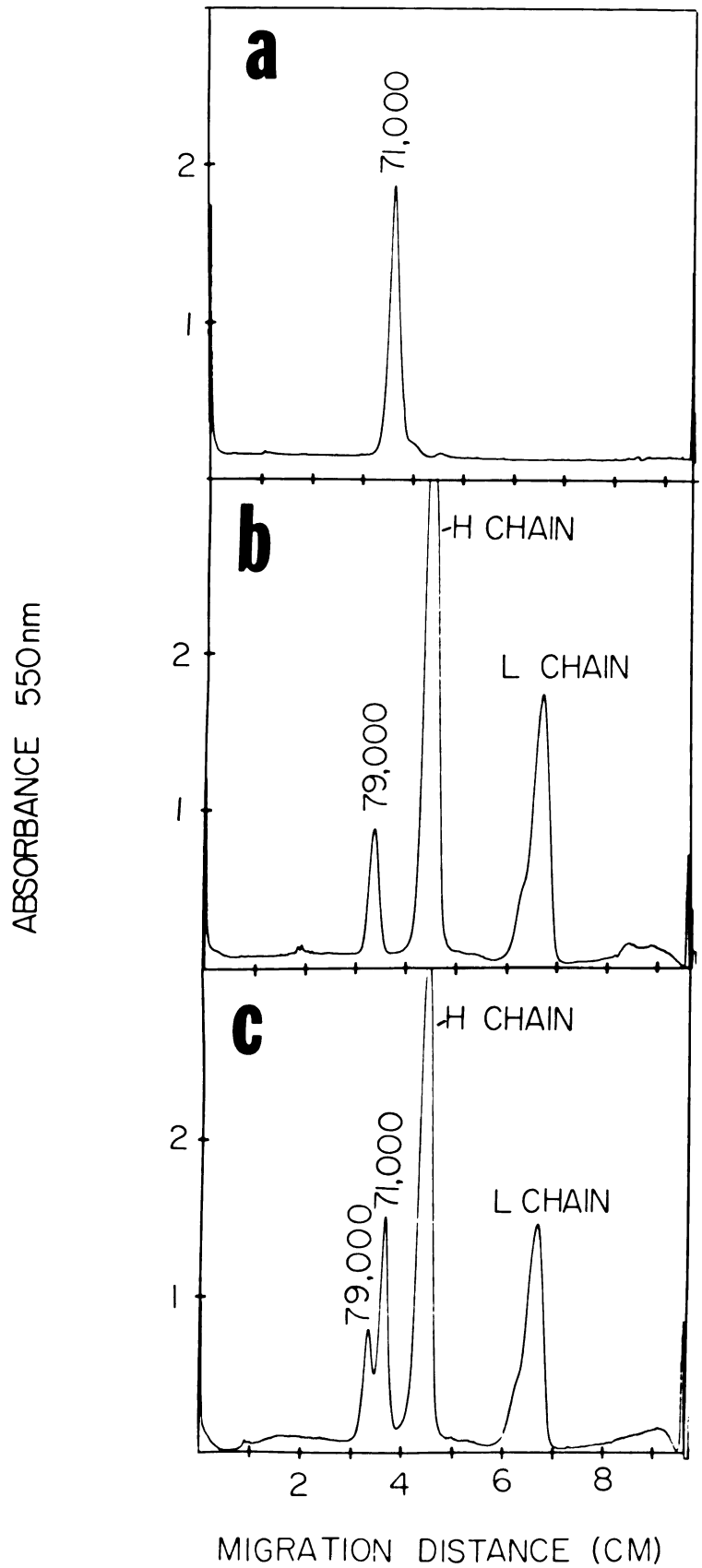
a
71,000
b
H CHAIN
L CHAIN
79,000
c
H CHAIN
79,000
71,000
L CHAIN
ABSORBANCE 550nm
MIGRATION DISTANCE (CM)

Figure 16

between antibody to the bromelain reductase and detergent-solubilized PB-microsomes, and a mixture of these two samples. It can be seen that the bromelain reductase has an apparent molecular weight of 71,000 ± 500 (average and standard deviation of 5 determinations) in comparison to the 79,000 molecular weight of the "native" reductase. Thus by SDS-polyacrylamide gel electrophoresis analysis, the bromelain reductase has a molecular weight approximately 8,000 daltons smaller than the "native" reductase.

In actuality it could be argued that the only true conclusion which can be drawn from studies using SDS-polyacrylamide gel electrophoresis is that the polypeptide subunits of the bromelain reductase are 8,000 daltons smaller than those of the native enzyme. By comparison of the elution profile of the proteolytically solubilized enzyme on Sephadex G-100 and the molecular weight value obtained on SDS-polyacrylamide gels, however, it has previously been concluded that the bromelain enzyme is composed of a single polypeptide chain (98). The antigenic similarities of the "native" reductase and the proteolytically solubilized form and their similar diffusion properties in agar during Ouchterlony double diffusion analysis tends to argue the native reductase is also composed of a single polypeptide chain.

Ouchterlony Double Diffusion Analysis of the Precipitin Reactions Between the Antibody to Bromelain Reductase and Detergent-Solubilized Liver Microsomal Proteins From Control, PB-, and 3-MC-Pretreated Rats

Ouchterlony double diffusion analysis was next used to assess the possibility of using the antibody to the bromelain reductase from PB-microsomes to also immunoprecipitate the "native" reductase enzymes

present in the liver microsomes from control and 3-MC-pretreated rats. As can be seen in Figure 17, a single precipitin line formed between the antibody, the bromelain reductase, and each of the three types of detergent-solubilized microsomes. Since all the precipitin lines fused, a single identical component in each type of microsomes was being precipitated. It is most likely that in each case this was the "native" reductase. Pre-immune IgG would not form a precipitate with any of the samples.

Enzymatic Characterization of the Immunoprecipitates Formed Between the Antibody and Detergent-Solubilized Microsomal Proteins from the Livers of Control, PB-, and 3-MC-Pretreated Rats

To further identify the component immunoprecipitated by the antibody from each type of detergent-solubilized microsomes, each immunoprecipitate was assayed for NADPH-ferricyanide reductase activity (Table VIII). In this experiment addition of the antibody to the detergent solubilized microsomes inhibited between 75-78% of this activity. All of the residual activity, however, was found in the immunoprecipitate, suggesting that all "native" reductase from each type of microsome had been immunoprecipitated.

Comparison of the Molecular Weights of the "Native" Reductases Present in the Liver Microsomes from Control, PB-, and 3-MC-Pretreated Rats

Figure 18 compares the SDS-polyacrylamide gel electrophoresis protein profiles of the immunoprecipitate formed from antibody to bromelain reductase and detergent-solubilized liver microsomal proteins from control, PB-, and 3-MC-pretreated rats. Each

Figure 17. OUCHTERLONY DOUBLE DIFFUSION ANALYSIS OF THE PRECIPITIN REACTIONS BETWEEN ANTIBODY TO BROMELAIN-SOLUBILIZED NADPH-CYTOCHROME C REDUCTASE, THE BROMELAIN REDUCTASE, AND DETERGENT-SOLUBILIZED LIVER MICROSOMAL PROTEINS FROM CONTROL, PB-, AND 3-MC-PRETREATED RATS

Ouchterlony double diffusion analysis was performed as described in the Materials and Methods. The center well contains anti-NADPH-cytochrome c reductase IgG (120 µg of protein). The outer wells contain various antigens and are numbered clockwise from the top (No. 1). Wells 1 and 4 contain 180 µg of PB-microsomal protein (6 mg/ml in 0.015 M sodium phosphate buffer, pH 8.0 containing 2% sodium deoxycholate). Well 2 contains 360 µg of 3-MC-microsomal protein (12 mg/ml in phosphate buffer containing 4% sodium deoxycholate). Well 3 contains 360 µg of control-microsomal protein (12 mg/ml in phosphate buffer containing 4% sodium deoxycholate). Well 6 contains 3 µg of Bromelain-reductase (0.2 mg/ml in phosphate buffer). Well 5 contains 0.015 M sodium phosphate buffer, pH 8.0 containing 4% sodium deoxycholate.

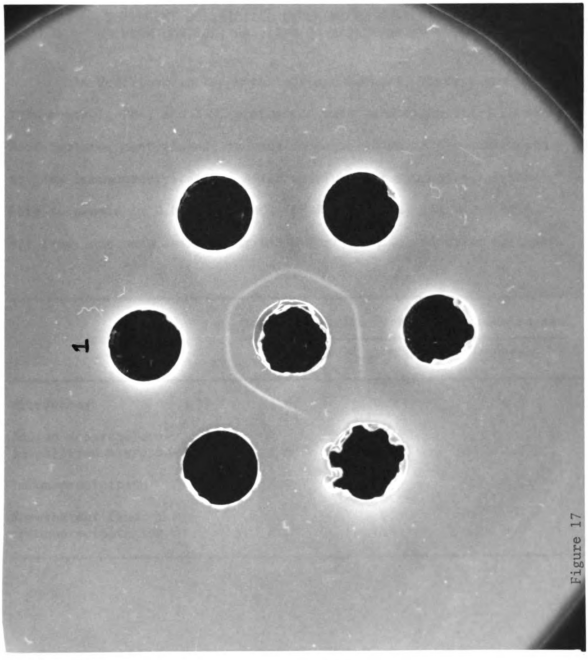

Figure 17

TABLE VIII

NADPH-FERRICYANIDE REDUCTASE ACTIVITIES IN THE IMMUNOPRECIPITATES FORMED BETWEEN ANTIBODY TO BROMELAIN REDUCTASE AND DETERGENT-SOLUBILIZED LIVER MICROSOMAL PROTEINS FROM CONTROL, PB-, AND 3-MC-PRETREATED RATS

As described in the Materials and Methods, liver microsomes from control, PB-, and 3-MC-pretreated rats were solubilized in sodium deoxycholate, centrifuged, and antibody was added to the supernatants to form immunoprecipitates. After washing, the immunoprecipitates were suspended in 0.05 M Tris-HCl (pH 7.5 at 25°C) and 10 mM EDTA. All fractions were assayed for NADPH-ferricyanide reductase activity.

Fraction	Total μmoles of ferricyanide reduced/min		
	PB	3-MC	Control
Microsomes	1.6	0.9	0.8
Sodium deoxycholate-solubilized microsomes	1.6	0.9	0.8
Immunoprecipitate	0.4	0.2	0.2
Supernatant from immunoprecipitation	0.0	0.0	0.0

Figure 18. A COMPARISON OF THE SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROTEIN PROFILES OF THE IMMUNOPRECIPITATES FORMED BETWEEN ANTIBODY PREPARED TO PURIFIED BROMELAIN-SOLUBILIZED NADPH-CYTOCHROME C REDUCTASE AND DETERGENT-SOLUBILIZED LIVER MICROSOMES FROM CONTROL, PB-, AND 3-MC-PRETREATED RATS

The scans on the left-hand side of this figure compare the profiles obtained for immunoprecipitates formed between reductase antibody and detergent-solubilized liver microsomes from control, PB-, and 3-MC-pretreated rats while the scans on the right-hand side compare the profiles obtained when mixtures of these samples were elctrophoresed on a single gel. Molecular weight markers, based on R_f values, were determined independently for each gel.

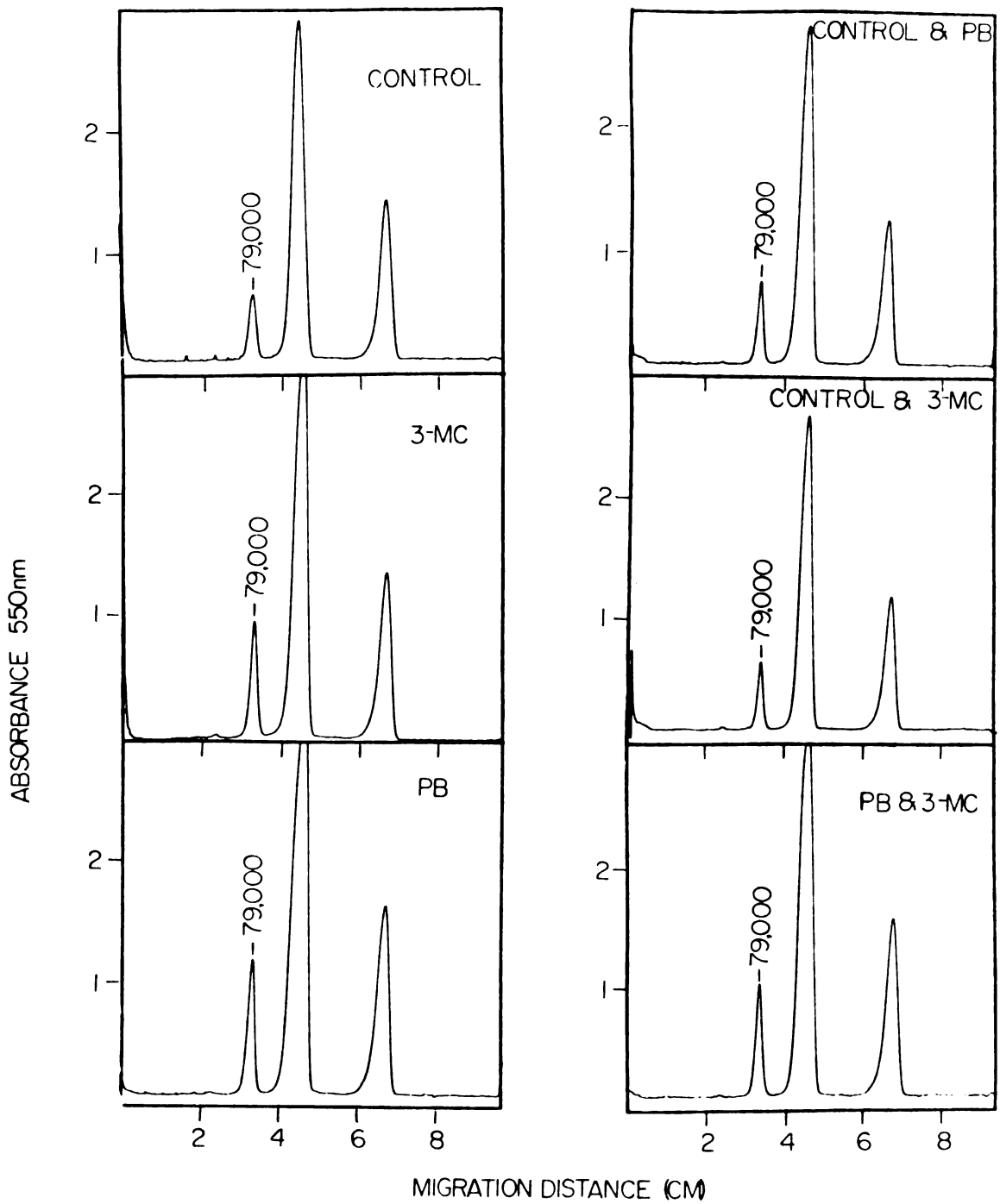
CONTROL
3-MC
PB
CONTROL & PB
CONTROL & 3-MC
PB & 3-MC
79,000
ABSORBANCE 550nm
MIGRATION DISTANCE (CM)

Figure 18

immunoprecipitate contains three protein constituents, one of molecular weight 79,000, one of molecular weight 52,000, and the other of molecular weight 25,000. The constituents of molecular weights 52,000 and 25,000 have previously been shown to be derived from the antibody. It appears from this experiment, that the "native" reductases from each type of microsomes have identical molecular weights. Even in mixing experiments in which combinations of two different immunoprecipitates were electrophoresed on a single gel, no difference could be detected in the molecular weights of the "native" reductases (Figure 18, right column of scans).

Discussion

From the results presented in this chapter it can be concluded that the "native" NADPH-cytochrome c reductase enzymes present in the liver microsomes from control, PB-, and 3-MC-pretreated rats appear to be identical both on the basis of their antigenic similarity and their apparent molecular weights on SDS-polyacrylamide gels. This conclusion concurs with those from other studies in which the trypsin-solubilized enzymes from liver microsomes of control and PB-pretreated rats have been shown to be immunologically identical and behave similarly during chromatography on Sephadex G-100 or DEAE-celluclose (58). Furthermore, other investigators have demonstrated that antibody to trypsin-solubilized reductase from PB-pretreated rats inhibits the reductase activity to the same degree in the liver microsomes from control, PB-, or 3-MC-pretreated rats (53,58), again suggesting that the reductases are identical. The one anomalous finding, however, is that by Lu *et al.* (73) in which it has been shown that "native" NADPH-cytochrome c

reductases partially purified from detergent-solubilized PB- and 3-MC-microsomal proteins appear to have a role in determining substrate specificity in reconstituted microsomal drug hydroxylation systems. This would not be expected if the reductase from the two types of microsomes were identical. Since the reductase preparations used in those studies were not pure, however, the observations made by Lu et al. (73) may have resulted from a contaminant in the preparations. Alternatively, the "native" reductases in the three different types of microsomes may differ in some very subtle manner which cannot be detected by studying their immunochemical or physical properties. Clearly, it will be necessary to isolate the "native" reductase from the three types of microsomes to differentiate between these possibilities.

It appears by SDS-polyacrylamide gel electrophoresis analysis that "native" NADPH-cytochrome c reductase has a molecular weight slightly larger than its proteolytically solubilized form. This suggests a structure for the "native" enzyme which is very similar to those already described by Strittmatter and co-workers for two other microsomal enzymes, NADH-cytochrome b_5 reductase (33) and cytochrome b_5 (34). These enzymes are amphipathic proteins--that is, they consist of a single polypeptide chain containing a large hydrophilic segment which contains the active site of the enzyme and is exposed to the cytoplasm of the cell and a smaller hydrophobic "tail" segment which interacts with the phospholipids of the microsomal membrane. Proteases cleave the hydrophilic portion of these proteins from the membrane while leaving behind the hydrophobic tail. If this is also the case with NADPH-cytochrome c reductase, this enzyme would appear to have a

tail of approximately 70 amino acids on the basis of the 8,000 dalton molecular weight difference of the native and proteolytically solubilized enzymes, determined in this study by SDS-gel electrophoresis. In actuality, the molecular weight difference may be greater, since Spatz and Strittmatter (33) have reported that SDS-gel electrophoresis gives a 20% underestimation of the molecular weight of the amphipathic NADH-cytochrome b_5 reductase. They attribute this disparity to the ability of the hydrophobic "tail" of this protein to bind extra SDS, increasing its mobility during electrophoresis and therefore decreasing its apparent molecular weight. If this is also the case with NADPH-cytochrome c reductase, the actual molecular weight of the "native" protein would be larger than 79,000.

The results of the studies presented in this chapter are consistent with the view that a single form of the enzyme NADPH-cytochrome c reductase is present in the microsomes from control, PB-, and 3-MC-pretreated rats. This suggests that the different hydroxylation activities found in these microsomes are not the result of multiple mixed-function oxidase electron transport chains. Therefore multiplicity must lie entirely within the oxygen- and substrate-binding component of the chain, cytochrome P_{450}. In the next two chapters, the role of this cytochrome is further investigated.

CHAPTER THREE

MULTIPLICITY OF CYTOCHROME P_{450} HEMOPROTEINS IN RAT LIVER MICROSOMES

Abstract

Cytochrome P_{450} is thought to be a major microsomal protein constituent having a molecular weight of approximately 50,000. Therefore the existence of multiple forms of this cytochrome in rat liver microsomes and their differential inducibility by PB- and 3-MC-pretreatment of rats could be investigated by SDS-polyacrylamide gel electrophoresis. Using this technique the 50,000 molecular weight protein components of the liver microsomes from control and PB- or 3-MC-pretreated rats were compared. It was observed that 3-MC-pretreatment induced a protein of slightly higher molecular weight than 50,000 (53,000) while PB induced a protein(s) of slightly lower molecular weight. The induced proteins co-purified with cytochrome P_{450} fractions prepared from the three types of microsomes. A method was developed by which benzidine and H_2O_2 could be used to stain for the peroxidase activity of cytochrome P_{450} hemoproteins on SDS-gels. Three hemoproteins were observed in rat liver microsomes using this technique and they have molecular weights of 53,000, 50,000, and 45,000. 3-MC appeared to induce the 53,000 dalton hemoprotein while PB induced the 45,000 dalton hemoprotein. These hemoprotein were also

present in partially purified fractions of this cytochrome from the three types of microsomes. These results suggest that multiple cytochrome P_{450} hemoproteins are present in rat liver microsomes.

The spatial position in the microsomal membrane of the proteins induced by PB and 3-MC was then investigated by combining the techniques of SDS-polyacrylamide gel electrophoresis and lactoperoxidase-catalyzed protein iodination. The liver microsomes isolated from rats pretreated with these compounds incorporate more ^{125}I into proteins of 50,000 molecular weight than do control microsomes. This suggests that the proteins induced by PB and 3-MC may be inserted onto the exterior of the microsomal membrane.

Introduction

For several years, investigations conducted using whole microsomal suspensions have suggested that different spectral forms of cytochrome P_{450} appear to be present in the liver microsomes isolated from control and PB- or 3-MC-pretreated rats. For example, in 1966 Imai and Sato (151) observed that a reduced difference spectrum of the cytochrome P_{450} in control microsomes, assayed in the presence of the ligand, ethyl isocyanide, exhibited two absorption maxima, one at 430 nm and the other at 455 nm. Subsequently Sladek and Mannering (152) reported that PB-pretreatment of animals caused an identical increase in the levels of both of these peaks while 3-MC-pretreatment only increased the level of the 455 nm peak. These results were interpreted to suggest that two forms of cytochrome P_{450} existed in microsomes and showed differential inductibility by PB and 3-MC. The

next year this idea was further substantiated when Alvares et al. (153) showed that the reduced CO-difference spectra of microsomes from control and PB- or 3-MC-pretreated rats also exhibited different absorbance maxima. The cytochrome assayed in the liver microsomes from control and PB-induced rats had an absorbance maximum at 450 nm while the maximum for the cytochrome in the microsomes from 3-MC-pretreated animals was at 448 nm. (Hence the cytochrome present in the liver microsomes from control and PB-pretreated rats is called cytochrome P_{450}, while that present in the microsomes from 3-MC-pretreated rats is termed cytochrome P_{448}.) Comparisons of substrate-binding spectra (154) and absolute spectra (66) of the cytochromes present in the liver microsomes from control and PB- or 3-MC-pretreated rats also suggested the presence of two forms of this cytochrome in these microsomes. Evidence other than spectral data has also supported this hypothesis. Thus, Levin and Kuntzman (155) reported a biphasic decay in the degradation of cytochrome P_{450} heme in microsomes, suggesting that two pools of this cytochrome exist in microsomes. They further noted that administration of PB increased the level of the component responsible for the fast turnover of heme while 3-MC-pretreatment induced the level of the component with the slow heme turnover.

In the past few years, more refined studies using detergent-solubilized microsomal fractions have also provided evidence which suggests that multiple forms of cytochrome P_{450} may exist in rat liver microsomes. For example, Lu and co-workers (73,74) have partially purified cytochrome P_{450} fractions from detergent-solubilized liver microsomes isolated from control and PB- and 3-MC-pretreated rats and

shown that each cytochrome P_{450} fraction appears to have different substrate specificities when assayed in a solubilized reconstituted microsomal hydroxylation system. Those hydroxylation activities normally induced in liver microsomes by PB-pretreatment of animals appeared to be catalyzed most effectively by the cytochrome fraction prepared from microsomes isolated from PB-pretreated rats. Similarly, those activities normally induced in liver microsomes by pretreatment of animals with 3-MC, were most effectively catalyzed by a cytochrome P_{448} fraction isolated from microsomes prepared from a rat pretreated with this compound. In addition, they discovered that cytochrome P_{450} fractions prepared from control microsomes appeared to have a different substrate specificity than the cytochrome fractions isolated from either the liver microsomes of 3-MC- or PB-pretreated rats. Thus on the basis of these studies using cytochrome P_{450}-enriched fractions from the liver microsomes of control and PB- or 3-MC-pretreated rats, it was suggested that not only did multiple catalytic forms of cytochrome P_{450} appear to exist in microsomes, but there appeared to be three forms of this cytochrome rather than two, as had been suggested by earlier work.

The existence of three forms of cytochrome P_{450} was also suggested by the results of another type of fractionation study conducted on detergent-solubilized microsomal proteins by Comai and Gaylor (156). In this study, protease-treated microsomes were solubilized with sodium deoxycholate and then subjected to DEAE-cellulose chromatography. This procedure resulted in the separation of three fractions of microsomal proteins which appeared to contain spectrally distinct forms of cytochrome P_{450}. The three forms of this

cytochrome were distinguished on the basis of their binding constants for cyanide and octylamine, as assayed by difference spectroscopy in the presence of these ligands. PB administration to rats was shown to induce one form while 3-MC-pretreatment induced a second. The third form could also be induced preferentially by ethyl alcohol. Thus, these observations too suggested that multiple forms of cytochrome P_{450} may exist in microsomes, but because the three spectrally distinct forms of this cytochrome observed in this study were separated from protease-treated microsomes, the possibility also existed that they may have resulted from proteolytic degradation of a single form of the cytochrome.

In any case, it is becoming an increasingly popular view that multiple forms of cytochrome P_{450} may be present in rat liver microsomes. All evidence for this proposal, however, has been obtained indirectly, because methods are not yet available to purify the cytochrome(s) free from other microsomal proteins. Thus, in actuality, little is known about the mechanism by which the apparently different spectral and catalytic forms of this cytochrome result in microsomes. It may be that different cytochrome P_{450} hemoproteins are present in microsomes and are preferentially induced by pretreatment of animals with various lipophilic compounds. Alternatively, only one cytochrome P_{450} hemoprotein may be present in microsomes and the spectral and catalytic properties of this hemoprotein may then be modified by other membrane constituents directly associated with the hemoprotein (i.e., perhaps a "regulatory" polypeptide or phospholipid?). In the studies presented in this chapter, the technique of SDS-polyacrylamide gel electrophoresis was used to further study this question.

Since it has been suggested by others that cytochrome P_{450} has a molecular weight of approximately 50,000 (61,66,67) and comprises 5 to 20% of the total microsomal protein (67,68), it was reasoned that the induction of this protein should be observable on SDS-gels of total microsomal protein. Furthermore, if multiple cytochrome P_{450} hemoproteins exist in microsomes and PB and 3-MC induce different hemoproteins, one should observe the induction of different proteins on SDS-gels run of liver microsomal proteins after rats are pretreated with these compounds. The results of the studies presented in this chapter demonstrate that PB and 3-MC do indeed induce different major microsomal proteins which are observable in the 50,000 molecular weight region of SDS-gels. These induced proteins are shown to co-purify with cytochrome P_{450}, suggesting their association with this cytochrome. Also they appear to be hemoproteins on the basis of their ability to be stained in SDS-gels with benzidine and H_2O_2. Furthermore, because the induction of major microsomal polypeptides after PB- and 3-MC-pretreatment of animals could be observed in SDS-polyacrylamide gels, it was reasoned that it might be possible to determine the position of these induced proteins in the microsomal membrane. Such knowledge would, in turn, allow a better understanding of how the proteins induced by PB- and 3-MC-pretreatment are incorporated into a pre-existing membrane. This information could be obtained by using the technique of lactoperoxidase-catalyzed protein iodination, developed in chapter one, to ^{125}I-label those proteins exposed to the exterior of the liver microsomal membranes isolated from control and PB- or 3-MC-pretreated rats. If the 50,000 molecular weight proteins induced by PB- and 3-MC-pretreatment of rats are located on the exterior of

the membrane, more ^{125}I should be incorporated into proteins of this molecular weight in the liver microsomes from PB- and 3-MC-pretreated rats than in the microsomes from control animals.

Materials and Methods

Materials

Male Sprague-Dawley rats weighing between 75-100 g were obtained from Spartan Research Animals, Inc., Haslett, Michigan.

Sodium cholate was obtained from the Schwarz-Mann Division of Becton Dickinson and Company, Orangeburg, New York. Benzidine-HCl was obtained from Merck and Company, Inc., Rahway, New Jersey. Purified Cytochrome P_{450} (cam) from Pseudomonas putida was the gift of Drs. Karl Dus and I. C. Gunsalus of the University of Illinois, Urbana, Illinois.

The sources of other reagents have been listed in chapters one and two.

Drug Pretreatment of Animals

The procedure for drug pretreatment of rats with PB and 3-MC was described in chapter two.

Isolation of Microsomal Membranes

The isolation and storage procedure used for microsomal membranes was described in chapter one. In some cases, isolated membranes were washed with 0.3 M sucrose containing 0.1 M sodium pyrophosphate, pH 7.5, also as described in that chapter.

Partial Purificiation of Cytochrome P_{450} Fractions From Liver Microsomes of Control and PB- or 3-MC-Pretreated Rats

The isolation procedure described by Levin et al. (66) was followed. Unless otherwise stated all procedures were carried out at 0-4°C. Liver microsomes, which had been stored in 0.05 M Tris-HCl, pH 7.5 containing 50% glycerol, were suspended to a protein concentration of 1-2 mg/ml in 1.15% KCl containing 10 mM EDTA, pH 7.5 and centrifuged at 105,000 x g for 90 minutes. The resulting microsomal pellets were resuspended to a protein concentration of 30-40 mg/ml in 0.25 M sucrose. Each 800-1000 mg of microsomal protein was then mixed with 14 ml of glycerol, 7 ml of 1 M potassium phosphate, pH 7.7, 0.7 ml of 0.1 M dithiothreitol, and 0.7 ml of 0.1 M EDTA, pH 7.5 and this mixture was then diluted to 61 ml with 0.25 M sucrose. The mixture was sonicated using four, 15 second sonication bursts at the number 4 setting on a Branson Model S-125 Sonifier (4 ma, maximum power output). During sonication the temperature of the mixture was not allowed to rise above 8°C. Eight to 10 ml of 10% sodium cholate was then added, with stirring, to the mixture bringing the final concentration to 1 mg cholate per mg of protein. The mixture was then stirred on ice for an additional 20 minutes and centrifuged at 105,000 x g for 90 minutes. After centrifugation, the supernatant was fractionated with ammonium sulfate. These fractionations were performed by slowly adding solid ammonium sulfate to the saturation desired, stirring the mixture an additional 20 minutes, and then centrifuging at 27,000 x g (15K in refrigerated Sorvall RC 2-B centrifuge, SS34 head) for 20 minutes to pellet the insoluble material. Solid ammonium sulfate was added to 40% saturation (0.224 g/ml) and then to 50% saturation (0.058 g/ml

additional ammonium sulfate). The material pelleting between 40 to 50% saturation was resuspended to approximately 13 ml in 0.05 M potassium phosphate buffer, pH 7.7 and centrifuged at 160,000 x g for 60 minutes to remove insoluble material. The supernatant fraction was then dialyzed overnight against 2 liters of 0.02 M potassium phosphate buffer, pH 7.7 containing 20% glycerol, 10^{-4} M dithiothreitol, 10^{-4} M EDTA, and 0.1% sodium cholate. After dialysis, the material was centrifuged at 27,000 x g for 20 minutes and the supernatant (usually between 10-20 mg protein/ml) was diluted to 3-4 mg protein/ml with 0.02 M potassium phosphate buffer, pH 7.7 containing 20% glycerol, 10^{-4} M dithiothreitol, 10^{-4} M EDTA, and 0.2% sodium cholate. Solid ammonium sulfate was added to 43% saturation (0.25 g/ml) and then to 50% saturation (0.042 g/ml additional ammonium sulfate). The resulting precipitate from 43-50% saturation was dissolved in 2-4 ml of 0.005 M potassium phosphate, pH 7.7 containing 20% glycerol, 10^{-4} M dithiothreitol, 10^{-4} M EDTA, and 0.1% sodium cholate. It was dialyzed overnight against this same buffer. The dialyzed sample was then centrifuged at 27,000 x g for 20 minutes and the supernatant was divided into small aliquots which were frozen under N_2 in sealed vials. The final protein concentration usually ranged between 3-8 mg/ml.

1% SDS-Polyacrylamide Gel Electrophoresis

The procedure described in chapter one was used for studies employing this electrophoresis technique.

0.1% SDS-Polyacrylamide Gel Electrophoresis

For 0.1% SDS-polyacrylamide gel electrophoresis, 10 cm gels were prepared according to the method of Fairbanks et al. (106), except the SDS concentration in the gels and in the electrophoresis buffer was lowered to 0.1%. (Note: under these conditions the gels polymerize in 10-15 minutes, much faster than in the presence of 1% SDS.) Microsome samples were suspended to a protein concentration of 6 mg/ml in 1% SDS containing 7% sucrose, 10 mM Tris-HCl, pH 8.0 (at 25°C), 1 mM EDTA, and 10 μg/ml pyronin B tracking dye. Partially purified cytochrome P_{450} fractions were suspended to a protein concentration of 3 mg/ml and the sample was only made 0.5% with respect to SDS. The samples were not boiled prior to electrophoresis. They were instead immediately applied to pre-electrophoresed gels and electrophoresis was performed in the dark at 5°C using an electrophoresis apparatus with a cooling jacket. A voltage gradient of 5 V/cm was used (2 ma/tube). Electrophoresis took about 8 hours under these conditions. After electrophoresis the gels were either stained for protein with Coomassie blue, as described in chapter one, or for peroxidase activity with benzidine and H_2O_2 (157). Molecular weights were determined as described in chapter one for the 1% SDS-polyacrylamide gel electrophoresis sytem. Dithiothreitol (40 mM) was included in the samples containing molecular weight markers.

Use of Benzidine and H_2O_2 to Stain for Cytochrome P_{450} Hemoproteins

Immediately after 0.1% SDS-polyacrylamide gel electrophoresis, the gels were placed in 0.02 M Tris-HCl, pH 7.5 (at 25°C) containing

50% methanol for 30 minutes to lower the SDS concentration within the gels. The gels were then placed in 0.25 M sodium acetate, pH 4.7 containing 0.25% benzidine, 25% methanol, and 0.75% H_2O_2 (157). This solution was made fresh just prior to use and was prepared by dissolving the required amount of benzidine-HCl in methanol and then adding the other reagents. H_2O_2 was added to the solution last. Since benzidine is carcinogenic, all staining procedures were carried out using rubber gloves and in the hood. After placing the gels in the staining solution, color development took approximately 15 minutes. Because the gels became opaque during the staining procedure and the stain was not stable for long periods of time, the gels could not be spectrophotometrically scanned and had to be photographed immediately after staining.

Lactoperoxidase-Catalyzed Protein Iodination

This procedure was performed as described in chapter one. The iodination mixture contained 2 μC $^{125}I^-$/ml.

Enzyme Assays

Cytochrome P_{450} and P_{420} concentrations were assayed as described by Imai and Sato (62) assuming values of 91 and -11 cm^{-1} mM^{-1} for extinction coefficients between 450 nm and 490 nm for P_{450} and P_{420}, respectively, and -41 and 110 cm^{-1} mM^{-1} between 420 nm and 490 nm for P_{450} and P_{420}, respectively. A computer program was used in making these calculations.

Results

Comparison of the 1% SDS-Polyacrylamide Gel Electrophoresis Protein Patterns of the Rat Liver Microsomes from Control and PB- or 3-MC-Pretreated Rats

The 1% SDS-polyacrylamide gel electrophoresis protein patterns of the rat liver microsomes from control and PB- or 3-MC-pretreated rats are compared in Figure 19. It can be seen that pretreatment of animals with either PB or 3-MC induces major microsomal protein constituents which migrate in the 50,000 molecular weight region of the gels. Furthermore, the two compounds do not induce the same proteins in this region. 3-MC appears to induce a component of slightly higher molecular weight than 50,000 while PB induces components of slightly lower molecular weight. Since pretreatment of animals with the two compounds does not appear to induce protein constituents having molecular weights below 40,000, these proteins can be electrophoresed off the gel to better resolve the protein constituents having molecular weights of approximately 50,000 (Figure 20). By comparison, the R_f values of all the proteins resolvable in this region of the gels, 6 different proteins can be observed among the three types of microsomes. To establish that 6 different microsomal proteins are indeed resolvable in this region of the gel, a mixture of the proteins from the liver microsomes of 3-MC- and PB-pretreated rats was electrophoresed in a single gel. In Figure 21 it can be seen that 6 proteins were visualized in this experiment and their apparent molecular weights are presented in Table IX. From Figure 20, at least four of the proteins are common to all three types of microsomes and these have been labeled proteins 1, 2, 4, and 6. Protein 3 is induced by 3-MC-pretreatment of animals.

Figure 19. 1% SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROTEIN PROFILES OF THE RAT LIVER MICROSOMES FROM CONTROL AND PB- OR 3-MC-PRETREATED RATS

Microsomal membranes were isolated by differential centrifugation and washed with 0.3 M sucrose containing 0.1 M sodium pyrophosphate, pH 7.5 prior to electrophoresis. As an index of induction by PB and 3-MC, the cytochrome P_{450} (448) levels (nmoles/mg microsomal protein) in the three types of microsomes were: control, 1.2; PB, 3.4; and 3-MC, 1.7. Between 40-45 μg of microsomal protein was applied to each gel. The protein banding patterns were visualized by staining with Coomassie blue and scanning the gels at 550 nm using a Gilford Spectrophotometer. The arrows indicate the positions on the gels of the protein(s) induced by 3-MC and PB.

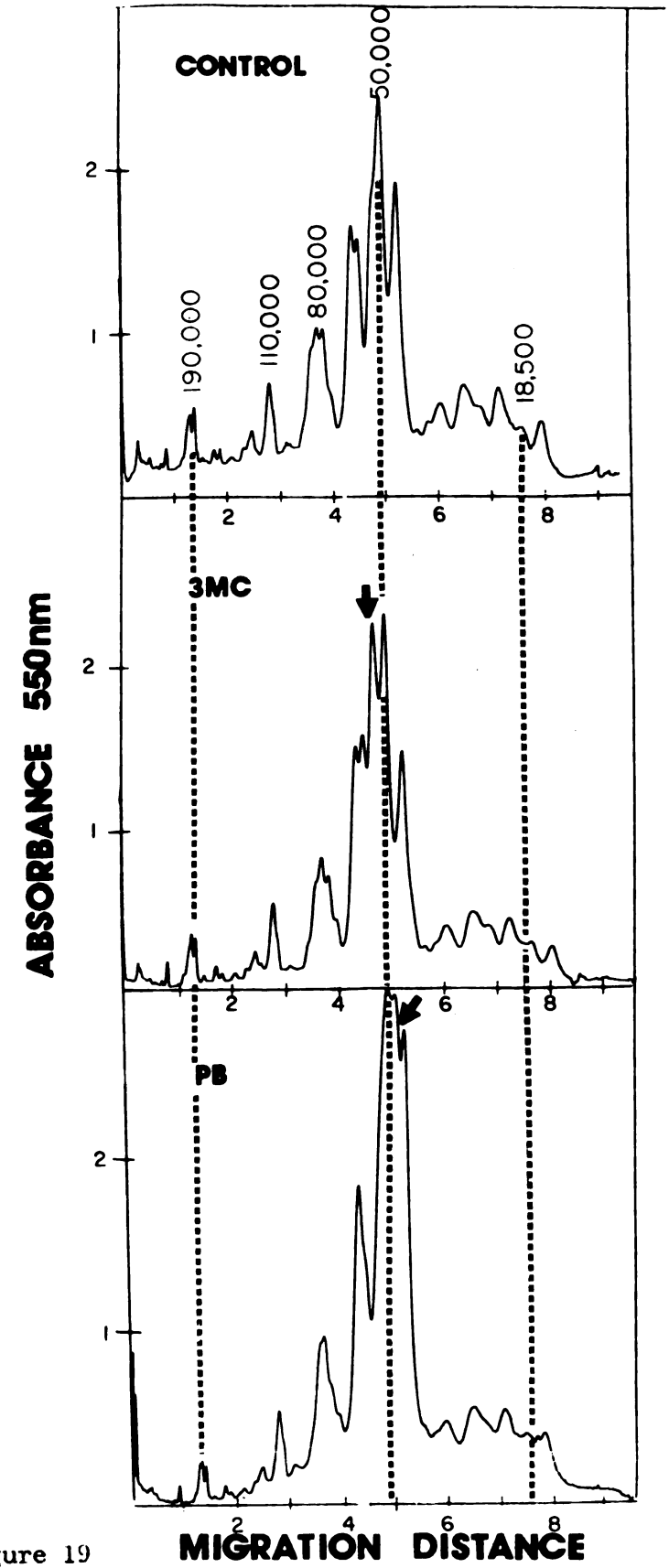
CONTROL
190,000
110,000
80,000
50,000
18,500
3MC
PB
ABSORBANCE 550nm
MIGRATION DISTANCE
1
2
2
4
6
8

Figure 19

Figure 20. 1% SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROFILES OF THE PROTEIN CONSTITUENTS HAVING MOLECULAR WEIGHTS LARGER THAN 40,000 WHICH ARE PRESENT IN THE RAT LIVER MICROSOMES FROM CONTROL AND PB-, AND 3-MC-PRETREATED RATS

Microsomal membranes were prepared as described in Figure 19. Electrophoresis was performed approximately 5-1/2 hours such that components having molecular weights lower than 40,000 would migrate off the gel. The protein patterns were visualized by staining with Coomassie blue and scanning the gels at 550 nm. Between 50-60 µg of protein was applied to each gel.

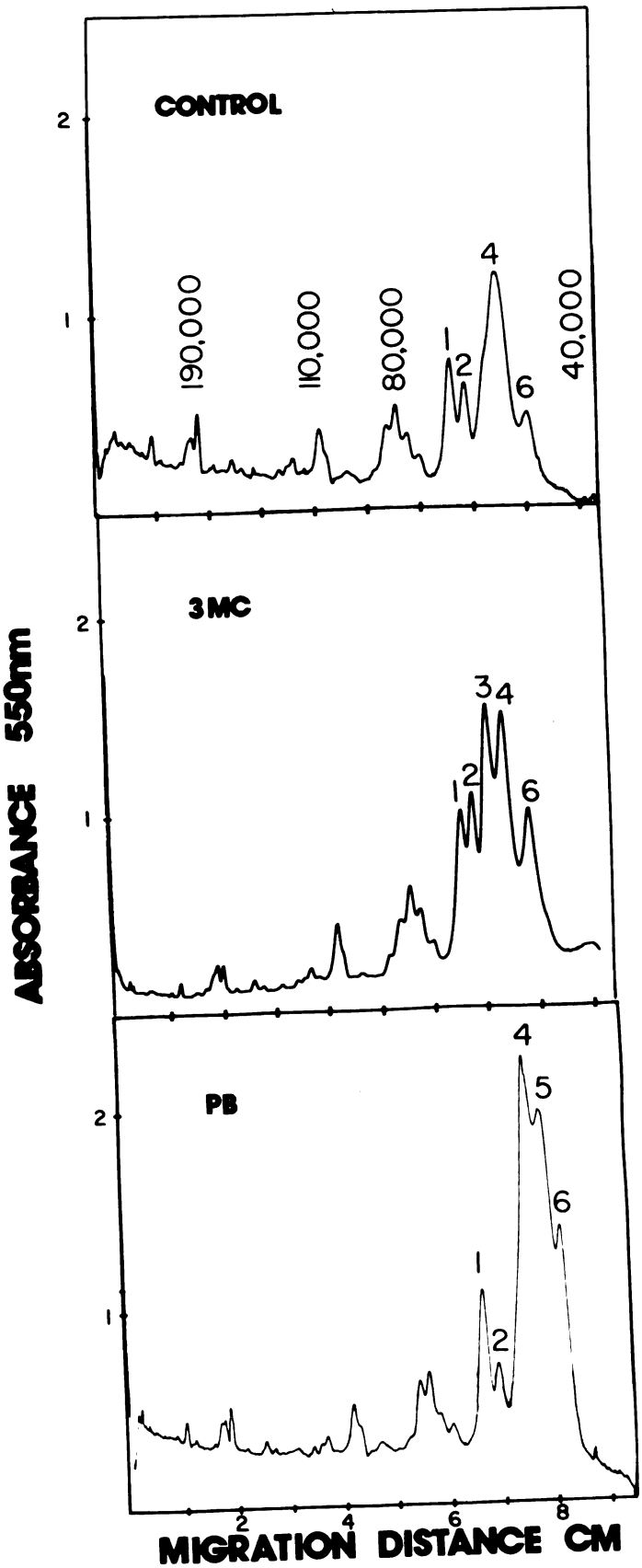
CONTROL
190,000
110,000
80,000
40,000
1
2
4
6
3MC
3
4
1
2
6
PB
4
5
6
1
2
ABSORBANCE 550nm
MIGRATION DISTANCE CM
2
4
6
8

Figure 20

Figure 20. 1% SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROFILES OF THE PROTEIN CONSTITUENTS HAVING MOLECULAR WEIGHTS LARGER THAN 40,000 WHICH ARE PRESENT IN THE RAT LIVER MICROSOMES FROM CONTROL AND PB-, AND 3-MC-PRETREATED RATS

Microsomal membranes were prepared as described in Figure 19. Electrophoresis was performed approximately 5-1/2 hours such that components having molecular weights lower than 40,000 would migrate off the gel. The protein patterns were visualized by staining with Coomassie blue and scanning the gels at 550 nm. Between 50-60 µg of protein was applied to each gel.

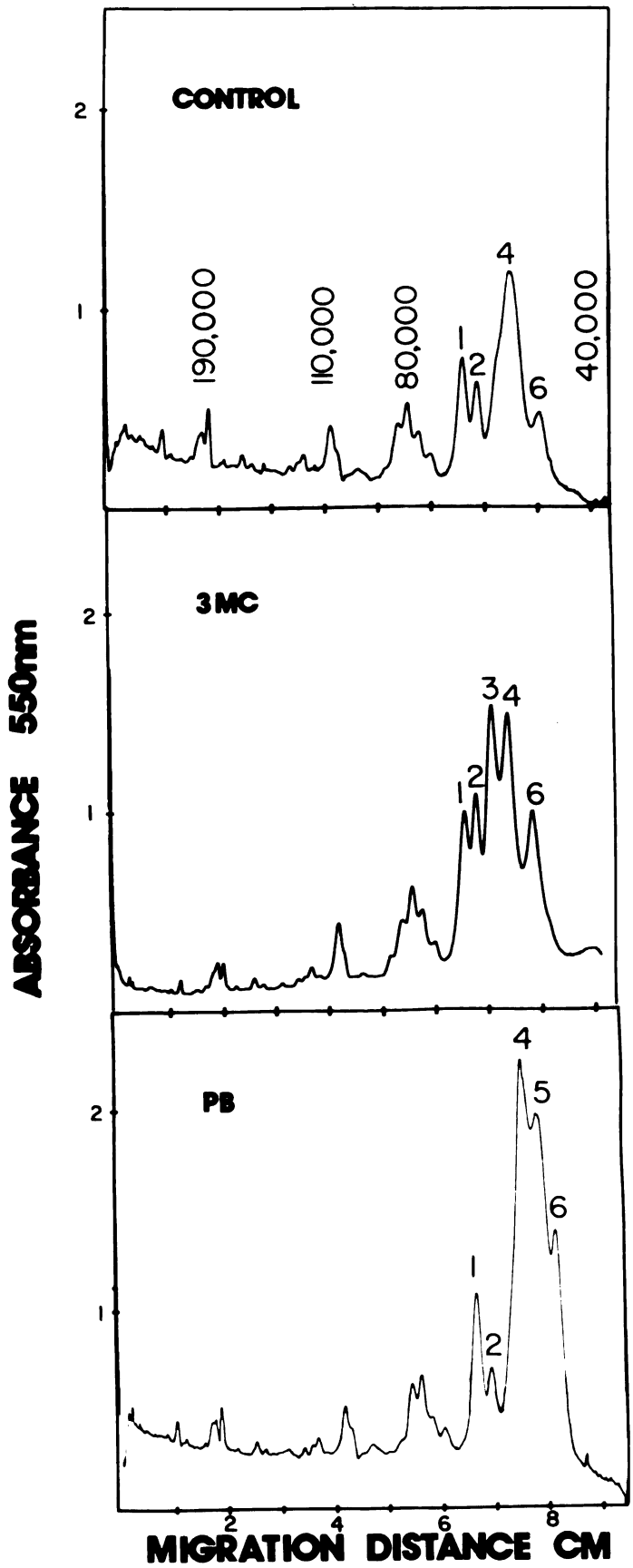
CONTROL
190,000
110,000
80,000
40,000
1
2
4
6
3MC
3
4
1
2
6
PB
4
5
6
1
2
ABSORBANCE 550nm
MIGRATION DISTANCE CM

Figure 20

Figure 21. 1% SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PATTERN OF THE PROTEIN CONSTITUENTS HAVING MOLECULAR WEIGHTS GREATER THAN 40,000 WHICH ARE PRESENT IN A MIXTURE OF THE LIVER MICROSOMES FROM 3-MC- AND PB-PRETREATED RATS

Microsomal membranes were prepared as described in the legend to Figure 19. Electophoresis was performed approximately 5-1/2 hours such that components having molecular weights below 40,000 migrated off the gel. Approximately 30 μg of protein from each type of microsome was applied to the gel. After electrophoresis the gel was stained with Coomassie blue and scanned at 550 nm.

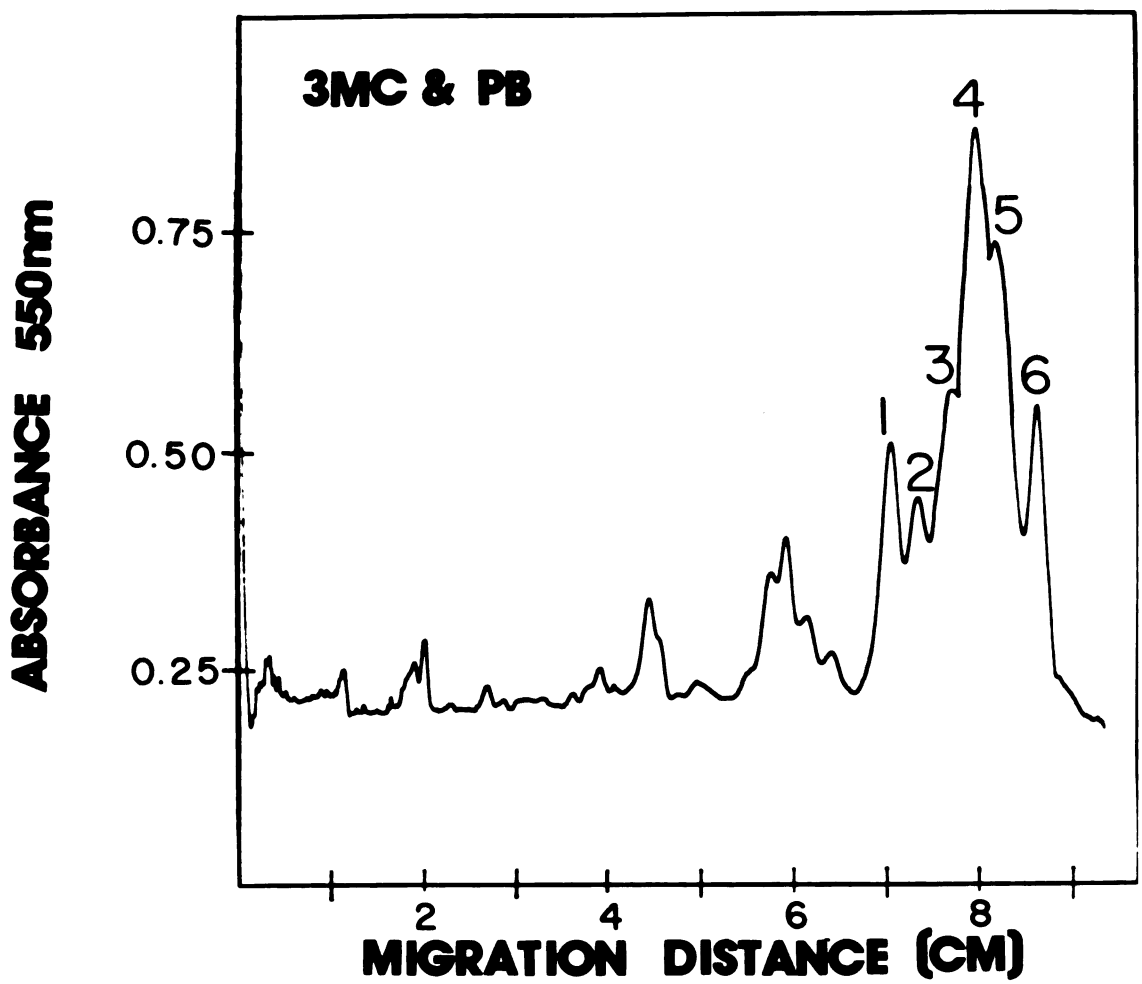
3MC & PB
ABSORBANCE 550nm
0.75
0.50
0.25
1
2
3
4
5
6
2
4
6
8
MIGRATION DISTANCE (CM)

Figure 21

TABLE IX

THE MOLECULAR WEIGHTS OF THE MAJOR PROTEIN CONSTITUENTS OF RAT LIVER MICROSOMES

The molecular weights of the major protein constitutents of rat liver microsomes were calculated as described by Weber and Osborn (108) from a standard curve prepared using the following proteins as molecular weight standards: β-galactosidase (130,000), bovine serum albumin (68,000), catalase (60,000), alcohol dehydrogenase (37,000), and carbonic anhydrase (29,000). The values reported for proteins 1, 2, 4, and 6 are the average and standard deviations from 9 determinations. The values for bands 3 and 5 are the averages and standard deviations from 3 determinations.

Protein	Molecular Weight
1	61,000 ± 1,000
2	58,000 ± 1,000
3	53,000 ± 1,000
4	50,000 ± 1,000
5	47,000 ± 2,000
6	45,000 ± 1,000

This component is probably also present in the liver microsomes from control and PB-pretreated animals but at levels at which it cannot be resolved from component 4. Proteins 2 and 6 may also be induced by 3-MC but to a much lesser extent than protein 3. The induction pattern after PB-pretreatment is more complex. It appears as if proteins 4, 5, and 6 are induced by pretreatment with this compound but since these proteins cannot be completely separated from one another during electrophoresis, this cannot be easily quantitated. Protein 5 appears to be induced to the greatest extent. This protein too is probably also present in the liver microsomes from control and 3-MC-pretreated rats but at levels at which it cannot be resolved from proteins 4 or 6.

1% SDS-Polyacrylamide Gel Electrophoresis Protein Profiles of the Liver Microsomes from Control and PB- or 3-MC-Pretreated Rats and the Cytochrome P_{450} Fractions Purified from the Microsomes

If the liver microsomal proteins induced by PB- or 3-MC-pretreatment of rats are associated with cytochrome P_{450}, they should co-purify with this cytochrome. To further explore this possibility, this cytochrome was partially purified from the liver microsomes of control and PB- or 3-MC-pretreated rats. The purification procedure used was similar to that described by Levin et al. (66). Their purification procedure consisted of two ammonium sulfate fractionations of sodium cholate-solubilized microsomes and a calcium phosphate gel extraction step. Since the calcium phosphate gel extraction did not appear to greatly increase the specific activity of the cytochrome and had a low yield, it was omitted from these studies. Table X summarizes

TABLE X

PARTIAL PURIFICATION OF CYTOCHROME $P_{450\ (448)}$ FROM THE LIVER MICROSOMES OF CONTROL AND PB- OR 3-MC-PRETREATED RATS

The purification procedure of Levin et al. (66) was followed, however the final calcium phosphate gel extraction was omitted. Cytochrome $P_{450\ (448)}$ and P_{420} concentrations were assayed and calculated as described in the Materials and Methods. The values in parentheses are those reported by Levin et al. (66).

Sample	Specific Activity $P_{450\ (448)}$ nmole/mg	Specific Activity P_{420} nmole/mg	Specific Activity Total Cytochrome nmole/mg	Fold Purification
Control				
Washed microsomes	1.0	0.2	1.2 (0.8)	. .
2nd Ammonium Sulfate (43-50%)	2.2	0.6	2.8 (2.0)	2.4 (2.6)
3-MC				
Washed microsomes	1.6	0.1	1.7 (1.4)	. .
2nd Ammonium Sulfate (43-50%)	3.5	0.5	4.0 (3.7)	2.4 (2.6)
PB				
Washed microsomes	3.1	0.3	3.4 (1.8)	. .
2nd Ammonium Sulfate (43-50%)	4.5	1.3	5.8 (4.4)	1.7 (2.4)

the specific activities of the cytochrome fractions purified from the liver microsomes isolated from control and PB- or 3-MC-pretreated rats. The final specific activities and fold purifications were similar to those reported by Levin et al. (66). Thus if the 50,000 molecular weight proteins induced by PB and 3-MC are associated with cytochrome P_{450}, these proteins should also appear in SDS-gels run of these partially purified cytochrome fractions.

Figure 22 compares the 1% SDS-polyacrylamide gel electrophoresis patterns of the proteins present in each partially purified cytochrome fraction with those present in the microsomes from which they were prepared. The major proteins in the cytochrome P_{450} preparations migrate in the 50,000 molecular weight region of the gels and, again, to best visualize components in this region of the gel, these samples were electrophoresed longer, allowing all components with molecular weights lower than 40,000 to migrate off the end of the gel (Figure 23). Using this procedure, four major proteins can be resolved among the three types of P_{450}. Again this can be seen most clearly by electrophoresing cytochrome fractions purified from both the liver microsomes of 3-MC- and PB-pretreated rats on a single gel (Figure 24). From Figure 23, protein 3 (53,000 daltons) which was induced in rat liver microsomes by pretreatment of rats with 3-MC is still present in the partially purified cytochrome fraction prepared from these microsomes. And, those proteins which appeared to be induced in the microsomes after pretreatment of rats with PB also co-purify with the cytochrome P_{450} preparation from these microsomes.

But even though it appeared from these studies that PB and 3-MC did induce different major microsomal proteins of molecular

Figure 22. 1% SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROTEIN PROFILES OF RAT LIVER MICROSOMES FROM CONTROL AND PB- OR 3-MC-PRETREATED RATS AND THE CYTOCHROME P_{450} FRACTIONS PURIFIED FROM THE MICROSOMES

Cytochrome P_{450} fractions were partially purified from rat liver microsomes as described in the Materials and Methods. The microsomal samples had been washed with 1.15% KCl containing 10 mM EDTA, pH 7.5. The cytochrome P_{450} levels in each sample are indicated in the upper right hand corner of the scan. Between 35-40 µg of total microsomal protein and 20-25 µg of protein from partially purified cytochrome P_{450} fractions were applied to a gel. The gels were stained with Coomassie blue and scanned at 550 nm.

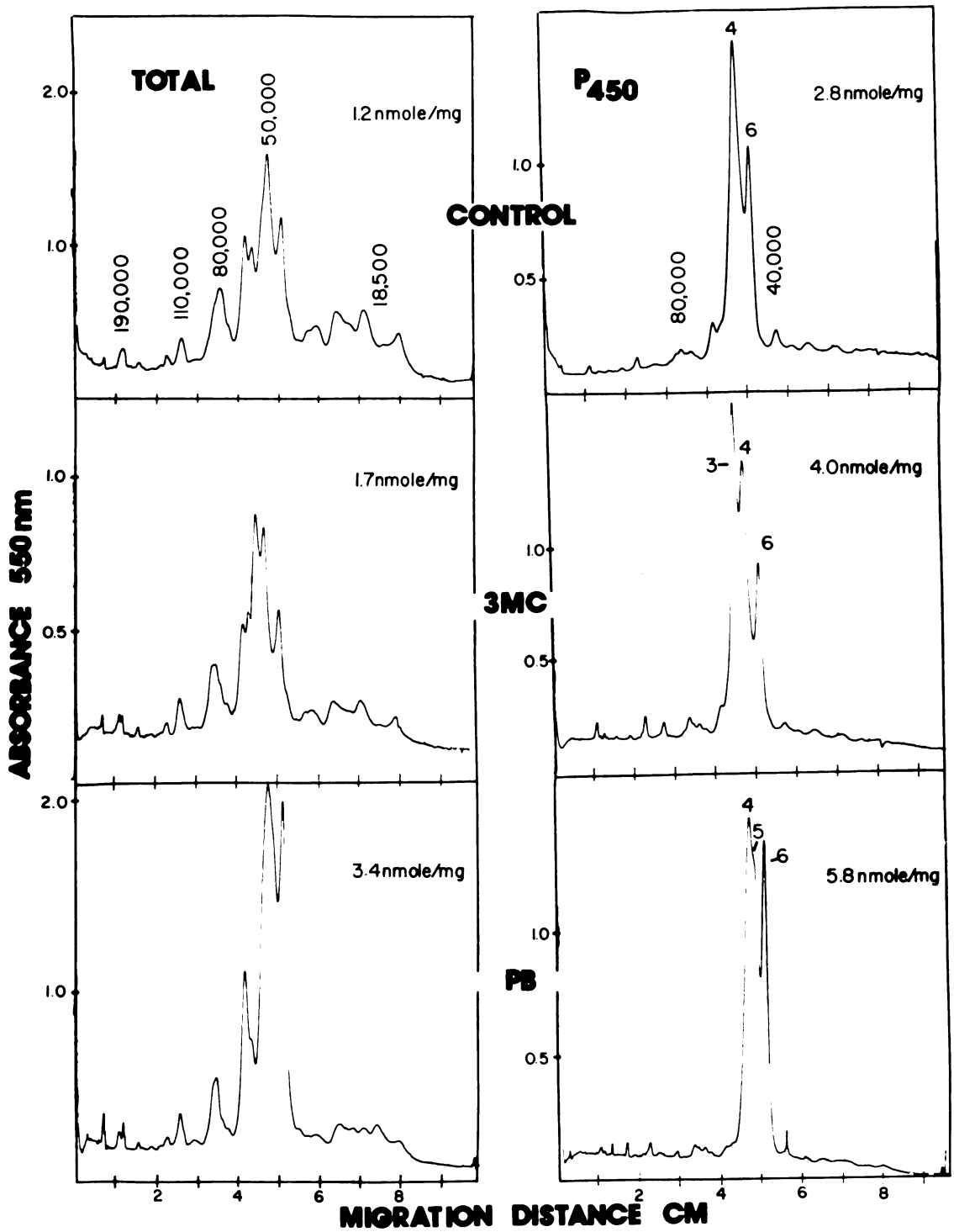
TOTAL
1.2nmole/mg
190,000
110,000
80,000
50,000
18,500
P450
2.8nmole/mg
80,000
40,000
CONTROL
1.7nmole/mg
4.0nmole/mg
3MC
3.4nmole/mg
5.8nmole/mg
PB
ABSORBANCE 550nm
MIGRATION DISTANCE CM

Figure 22

Figure 23. 1% SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROFILES OF THE PROTEIN CONSTITUENTS HAVING MOLECULAR WEIGHTS LARGER THAN 40,000 WHICH ARE PRESENT IN THE RAT LIVER MICROSOMES FROM CONTROL AND PB- OR 3-MC-PRETREATED RATS AND IN THE CYTOCHROME P_{450} FRACTIONS PURIFIED FROM THE MICROSOMES

Samples of rat liver microsomes (washed with 1.15% KCl containing 10 mM EDTA, pH 7.5) and partially purified cytochrome P_{450} fractions were electrophoresed for approximately 5-1/2 hours such that all protein constituents having molecular weights lower than 40,000 migrated off the gels. Between 50-60 µg of total microsomal protein and 30-40 µg of protein from the cytochrome fractions were applied to a gel. The cytochrome P_{450} level in the various samples is indicated in the upper left hand corner of the gel scan. Gels were stained with Coomassie blue and scanned at 550 nm.

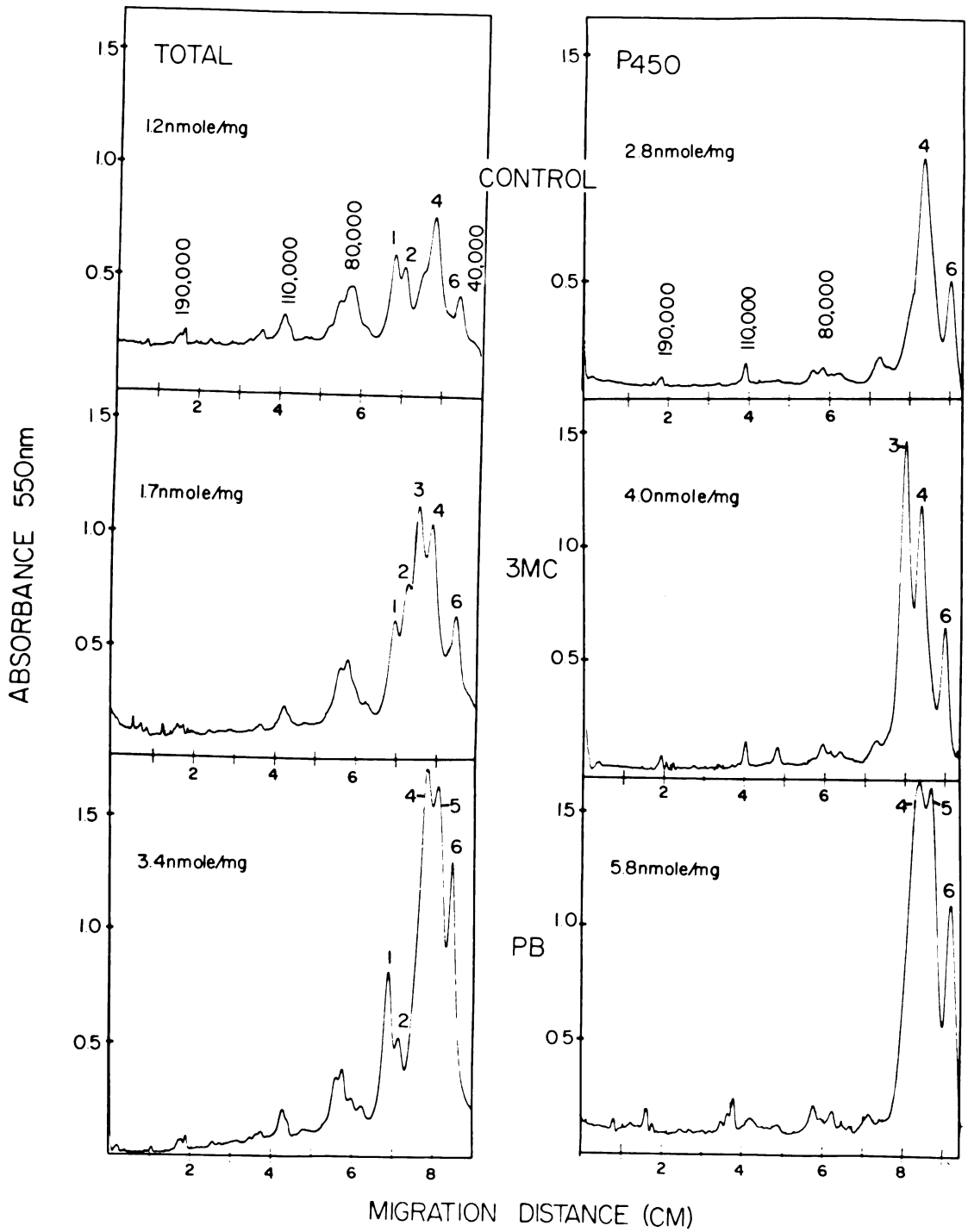
TOTAL
1.2nmole/mg
190,000
110,000
80,000
40,000
CONTROL
P450
2.8nmole/mg
1.7nmole/mg
4.0nmole/mg
3MC
3.4nmole/mg
5.8nmole/mg
PB
ABSORBANCE 550nm
MIGRATION DISTANCE (CM)

Figure 23

Figure 24. 1% SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PATTERN OF THE PROTEIN CONSTITUENTS HAVING MOLECULAR WEIGHTS GREATER THAN 40,000 WHICH CAN BE RESOLVED FROM A MIXTURE OF THE PARTIALLY PURIFIED CYTOCHROME $P_{450\ (448)}$ FRACTIONS ISOLATED FROM THE LIVER MICROSOMES OF 3-MC- AND PB-PRETREATED RATS

A mixture of the cytochrome $P_{450\ (448)}$ fractions isolated from the liver microsomes of PB- and 3-MC-pretreated rats was electrophoresed in a single gel. 20 µg of each cytochrome fraction was applied to the gel. Electrophoresis was performed such that components having molecular weights below 40,000 migrated off the gel. The gels were stained with Coomassie blue and scanned at 550 nm.

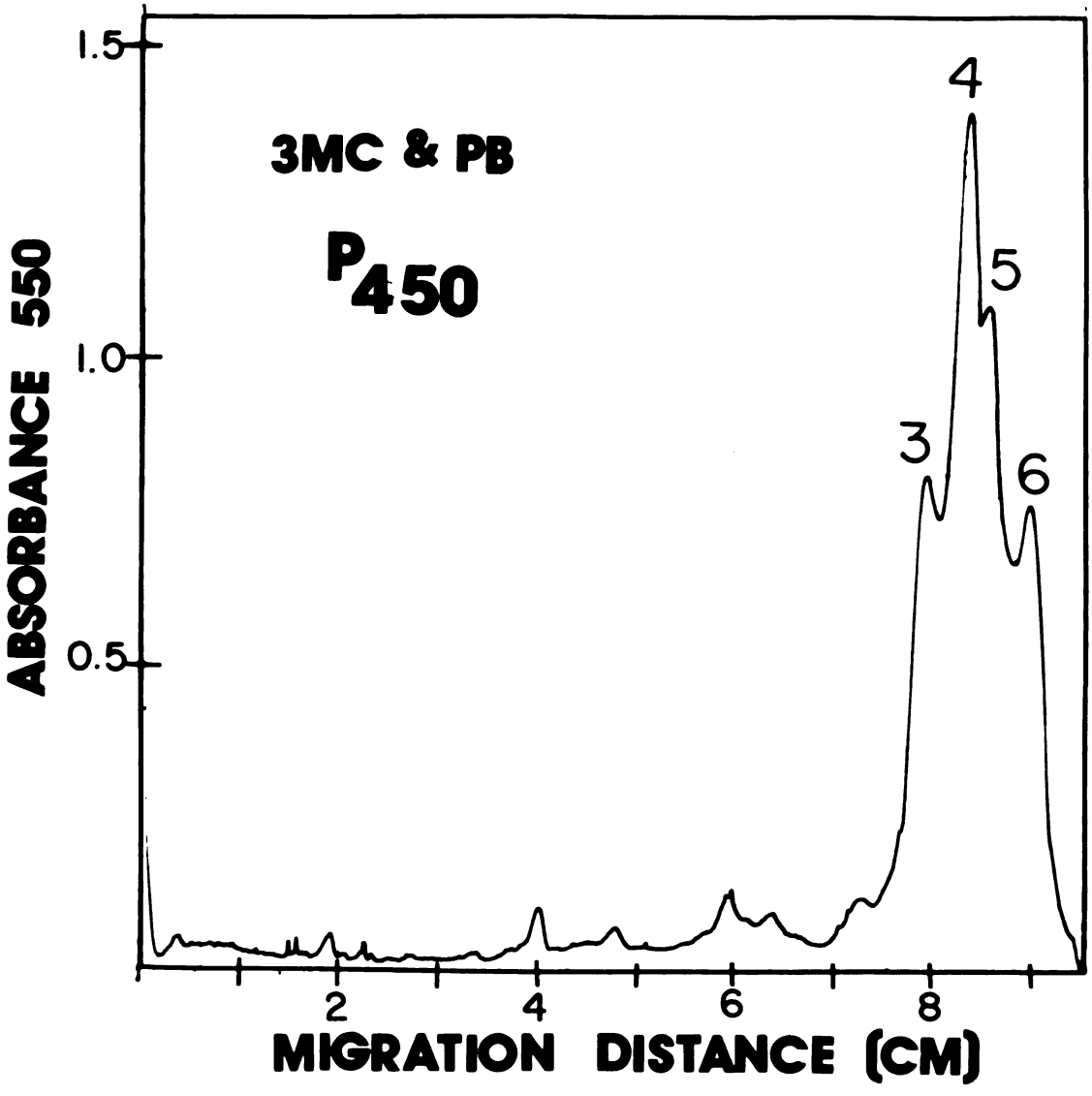
3MC & PB
P450
ABSORBANCE 550
1.5
1.0
0.5
2
4
6
8
MIGRATION DISTANCE (CM)
3
4
5
6

Figure 24

weight 50,000 and that these proteins co-purified with cytochrome P_{450}, it still remained to be proven that the induced proteins were actually hemoproteins. That is, these studies did not eliminate the possibility that there was only one cytochrome P_{450} hemoprotein present in microsomes and that the induced proteins were different "regulatory" proteins which modified the spectral and catalytic activities of the cytochrome. Experiments were therefore undertaken in order to identify the cytochrome P_{450} hemoprotein(s) on SDS-gels run of liver microsomal proteins.

Use of Benzidine and H_2O_2 to Stain for Cytochrome P_{450} Hemoprotein(s) on SDS-Polyacrylamide Gels

Several studies have suggested that under appropriate conditions, heme does not completely dissociate from cytochrome P_{450} hemoproteins in the presence of SDS. For example, Yu and Gunsalus (158) have shown that cytochrome P_{450} (cam), isolated from Pseudomonas putida, can be dialyzed against 1% SDS for 20 hours and lose no more than 5% of its heme as long as no sulfhydryl reagent is present in the buffer. Furthermore, Black and Bresnick (159) have shown that all of the heme is not removed from rat liver microsomal cytochrome P_{450} during SDS-polyacrylamide gel electrophoresis of ^{3}[H]-heme-labeled microsomes. Since use of ^{3}[H]-heme-labeled microsomes required the application of large amounts of microsomal protein to SDS-gels, however, that method was not sensitive enough to resolve multiple bands in the 50,000 molecular weight region of a gel. It did suggest though that if a sensitive staining technique to assay for cytochrome P_{450} hemoproteins

on SDS-gels could be developed, it might be possible to determine if the different induced proteins were indeed hemoproteins.

Since Hrycay and O'Brien (160,161) have shown that in SDS, cytochrome P_{450} is converted to cytochrome P_{420} which has peroxidase activity, a method was developed by which benzidine and H_2O_2 could be used to stain for this peroxidase activity (157) in SDS-gels. This method required that the SDS-concentration in the electrophoresis gels and buffer be lowered to 0.1% and that the sample be prepared for electrophoresis without boiling and in the absence of dithiothreitol. This prevented the complete dissociation of heme from cytochrome P_{420} during electrophoresis. Such modifications in the electrophoresis procedure slightly altered the protein banding pattern in the 50,000 molecular weight region of SDS-gels, as can be seen in Figure 25. Overall, the same induction pattern can be seen after PB- or 3-MC-pretreatment of rats, however. That is, 3-MC appears to induce a protein of molecular weight 53,000 (designated protein 2 in Figure 25) while PB induces proteins having molecular weights of 47,000 (protein 4) and 45,000 (protein 5). When similar gels were then stained with benzidine and H_2O_2, as described in the Materials and Methods, the patterns visualized in Figure 26 were obtained. Since the gels became opaque during the staining procedure, they could not be scanned and were therefore photographed. There appear to be three benzidine-staining bands in the 50,000 molecular weight region of the gels, where cytochrome P_{450} should migrate. In addition a band of material near the bottom of the gels, just above the tracking dye was stained with benzidine. This band is probably due to dissociated heme. The three stained components in the middle of the gel had molecular

Figure 25. 0.1% SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROTEIN PROFILES OF THE 50,000 MOLECULAR WEIGHT REGION OF GELS RUN ON LIVER MICROSOMES FROM CONTROL AND PB- OR 3-MC-PRETREATED RATS

Sucrose containing sodium pyrophosphate-washed microsomes from control, PB-, and 3-MC-pretreated rats were electrophoresed on 0.1% SDS-gels as described in the Materials and Methods section. 60 µg of microsomal protein was applied to each gel. After electrophoresis the gels were stained with Coomassie blue and the 50,000 molecular weight region was spectrophotometrically scanned at 550 nm. The upper scan is from the gel run on microsomes from 3-MC-pretreated rats; the middle from control rats, and the lower from PB-pretreated rats. Bands labeled 1, 2, 3, 4, and 5 have the following apparent molecular weights: 57,000 ± 1,000; 53,000 ± 1,000; 50,000 ± 1,000; 47,000 ± 1,000; and 45,000 ± 1,000. These molecular weight values are the averages and standard deviations from 6 determinations.

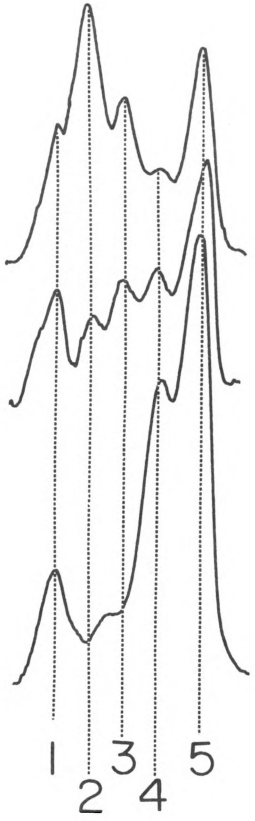

Figure 25

Figure 26. HEMOPROTEIN PROFILES OBTAINED BY STAINING 0.1% SDS-POLYACRYLAMIDE GELS OF LIVER MICROSOMES FROM CONTROL AND PB- OR 3-MC-PRETREATED RATS WITH BENZIDINE AND H_2O_2

Sucrose containing sodium pyrophosphate-washed microsomes were electrophoresed on 0.1% SDS-gels and stained with benzidine and H_2O_2 as described in the Materials and Methods. 100 µg of protein was applied to each gel. The upper-most hemoprotein has an apparent molecular weight of 53,000 ± 1,000; the middle, 50,000 ± 1,000; and the lowest, 45,000 ± 1,000. These molecular weight values are the averages and standard deviations from 6 determinations. The band of stained material immediately above the tracking due is probably dissociated heme.

Figure 26

weights of 53,000, 50,000, and 45,000. On the basis of these molecular weights these three hemoproteins would correspond to proteins 2, 3, and 5 observed in Figure 25.

It is possible to use Figure 26 to compare the relative amounts of the three hemoproteins in each type of microsome if it is assumed that the percentage of heme lost from each protein is the same. In this case, all three hemoproteins are of about equal concentration in control microsomes. The 53,000 dalton hemoprotein is induced by 3-MC-pretreatment while PB induces the 45,000 dalton hemoprotein (in the case of PB induction, the 45,000 dalton hemoprotein almost appears to be induced at the expense of the other hemoproteins). The induction pattern seen after benzidine-staining corresponds to that seen in Figure 25 after Coomassie blue staining and is also reminiscent of those described in Figures 19 and 20, using a slightly different gel system.

It should be noted that a control experiment was conducted to demonstrate that the multiple benzidine-staining bands observed on SDS-gels of total microsomal proteins were not the result of the dissociation of heme from a single hemoprotein and its rebinding to other microsomal proteins during the preparation of samples for electrophoresis. In this experiment, a purified preparation of cytochrome P_{450} (cam) from Pseudomonas putida was mixed with bovine serum albumin and electrophoresed on a SDS-gel. This was thought to be a good control because the characteristics of cytochrome P_{450} (cam) should be very analogous to those of the microsomal cytochromes and bovine serum albumin is known to have a strong affinity for heme (162,163), even though not itself a hemoprotein. It was observed in

this experiment that only cytochrome P_{450} (cam) stained with benzidine on the SDS-gels, indicating that binding of dissociated heme by non-hemoproteins was probably not a problem in these studies.

Hemoprotein Profiles of Partially Purified Cytochrome P_{450} Preparations Isolated from the Liver Microsomes of Control and PB- or 3-MC-Pretreated Rats

If the three hemoproteins, identified by SDS-polyacrylamide gel electrophoresis of microsomal proteins, are indeed cytochrome P_{450} hemoproteins, they should also be present in the partially purified preparations of this cytochrome which were isolated from control and PB- or 3-MC-microsomes. As can be seen in Figure 27, this was the case. When these preparations were electrophoresed on 0.1% SDS-gels and stained with benzidine, they appeared to contain the same hemoprotein composition as did the microsomes from which they were isolated. Thus the 53,000 dalton hemoprotein previously shown to be enriched in the microsomes from 3-MC-pretreated rats is also enriched in the partially purified cytochrome fraction prepared from it. Similarly, the 45,000 dalton hemoprotein previously shown to be induced in microsomes by PB-pretreatment is enriched in the cytochrome fraction prepared from it. This observation, then, helps confirm the identity of the microsomal hemoproteins.

As noted before in the case of whole microsomes, electrophoresis of these partially purified preparations on 0.1% SDS-gels, under the conditions needed to conduct benzidine-staining, modifies the Coomassie blue banding pattern previously seen on 1% SDS-gels. The profiles obtained in both systems are compared in Figure 28. Both show similar induction patterns, however, in that the cytochrome

Figure 27. HEMOPROTEIN PROFILES OF CYTOCHROME $P_{450\ (448)}$ FRACTIONS ISOLATED FROM THE LIVER MICROSOMES OF CONTROL AND PB- OR 3-MC-PRETREATED RATS

Partially purified cytochrome $P_{450\ (448)}$ fractions isolated from the three types of microsomes were electrophoresed on 0.1% SDS-polyacrylamide gels as described in the Materials and Methods section. These gels were then stained for hemoproteins using benzidine and H_2O_2. The gel on the left is of the cytochrome fraction purified from the liver microsomes isolated from a 3-MC-pretreated rat, the middle gel is of the cytochrome fraction from the liver microsomes of a control rat, and the gel on the right is of the cytochrome fraction isolated from the liver microsomes of a PB-pretreated rat. 45 µg of protein was applied to each gel. The upper most hemoprotein has an apparent molecular weight of 53,000 $\pm$ 1,000; the middle, 50,000 $\pm$ 1,000; and the lowest 45,000 $\pm$ 1,000. The band of stained material near the bottom of the gel is probably dissociated heme.

Figure 28. A COMPARISON OF THE COOMASSIE BLUE PROTEIN PATTERNS OBTAINED BY ELECTROPHORESING CYTOCHROME P_{450} FRACTIONS ISOLATED FROM THE LIVER MICROSOMES OF CONTROL AND PB- OR 3-MC-PRETREATED RATS ON 1% AND 0.1% SDS-POLYACRYLAMIDE GELS

Samples were prepared for electrophoresis in the two gel systems under the conditions described in the Materials and Methods section. 30 µg of protein was applied to each gel. The gel scans on the left-hand side are the protein banding patterns obtained in the 0.1% SDS-gel system while those on the right were obtained using the 1% SDS-gel system. The specific activity of cytochrome P_{450} (nmoles/mg protein) for the samples used were: control, 2.8; 3-MC, 4.0; and PB, 5.8.

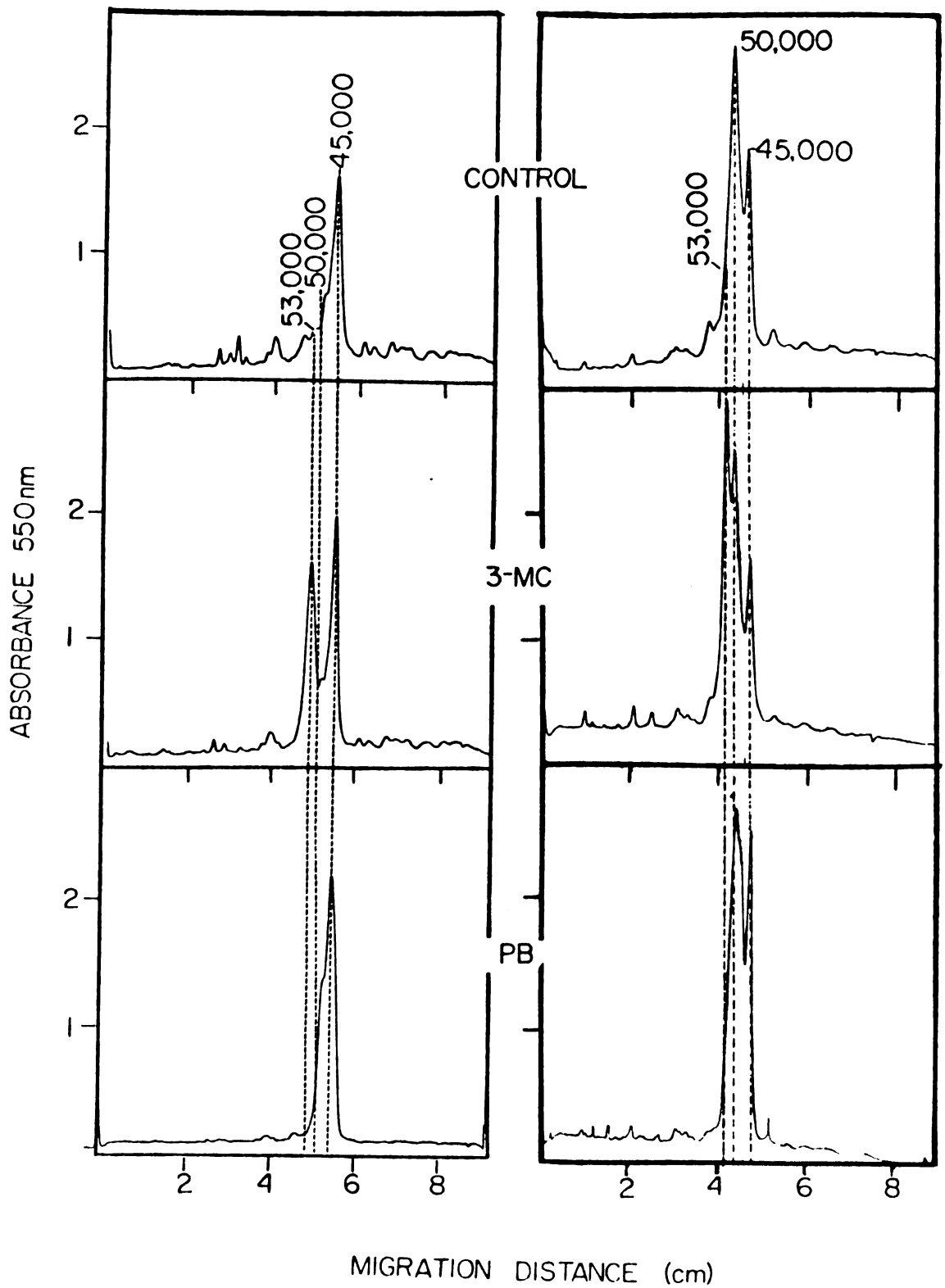
CONTROL
3-MC
PB
53,000
50,000
45,000
ABSORBANCE 550nm
MIGRATION DISTANCE (cm)

Figure 28

fraction isolated from 3-MC-pretreated rats is enriched in a 53,000 dalton component while that from PB is enriched in a 45,000 dalton component. And, in both cases, this induction pattern corresponds to that seen after benzidine-staining. But the two patterns differ in that other proteins present in these preparations migrate as polypeptides of molecular weight 45,000 on 0.1% SDS-gels and 50,000 on 1% SDS-gels. This difference in the migratory properties of the protein components was not due to the fact that the samples prepared for 1% SDS-gel electrophoresis had been boiled with dithiothreitol while those prepared for 0.1% SDS-gel electrophoresis had not. In fact it was found that the same profiles were obtained if the samples were prepared for electrophoresis in the presence or absence of this sulfhydryl reducing agent. The difference in patterns made it difficult to decide which of the proteins visualized by 1% SDS-gel electrophoresis were the hemoproteins. In all probability, the 53,000 dalton component seen by 1% SDS-gel electrophoresis to be induced by 3-MC-pretreatment (Figure 28, right hand column) corresponds to the hemoprotein of identical molecular weight observed on benzidine-stained gels (Figure 27), and in the next chapter evidence will be presented to suggest that the hemoprotein induced by PB-pretreatment also has an identical molecular weight (45,000) in both gel systems.

Comparison of the SDS-Polyacrylamide Gel Electrophoresis Protein and ^{125}I-Incorporation Profiles of the Liver Microsomes from Control and PB- or 3-MC-Pretreated Rats

Since the induction of major microsomal proteins by PB and 3-MC could be observed in the 50,000 molecular weight region of

SDS-gels, it was reasoned that it might be possible to use enzymatic iodination to study the position of these proteins within the microsomal membrane. Such studies could give some insight into how the proteins are inserted into a pre-existing membrane during the induction process. It was reasoned that because only membrane proteins located on the exterior of the membrane are ^{125}I-labeled by lactoperoxidase, if the induced proteins have an exterior location, the amount of ^{125}I incorporated into proteins which migrate in the 50,000 molecular weight region of SDS-gels should increase upon induction. As can be seen in Figure 29, the amount of ^{125}I incorporated into proteins in this region did increase in the induced microsomes. Because of the complexity of the protein banding pattern in this region of the gels, a direct comparison of the cpm of ^{125}I/absorbance at 550 nm ratios for the proteins cannot be made. A calculation can be made of the percent of ^{125}I incorporated into microsomal proteins having molecular weights between 40,000 to 60,000 daltons (Table XI), however, and by this criteria also, there is increased ^{125}I-incorporation into these proteins. Since after 3-MC-pretreatment of rats the induction pattern in the 50,000 molecular weight region of polyacrylamide gels is fairly simple, it is likely that the increased incorporation of ^{125}I in this region is due to the induction of the 53,000 molecular weight protein, identified as a hemoprotein. Because of the complexity of the PB induction pattern in this region and the resolution limitations involved in fractionating gels to determine the radioactivity profile of proteins of such close molecular weights, it is impossible to determine which protein(s) induced by PB is labeled with ^{125}I. In actuality it appears that PB-pretreatment induces other

Figure 29. A COMPARISON OF THE 1% SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROTEIN AND ^{125}I-INCORPORATION PROFILES OBTAINED FROM THE LIVER MICROSOMES OF CONTROL AND PB- OR 3-MC-PRETREATED RATS

The microsomes were washed with sucrose containing sodium pyrophosphate, iodinated, and prepared for electrophoresis. As an index of induction, the cytochrome $P_{450\ (448)}$ levels (nmoles/mg microsomal protein) in the three types of microsomes were: control, 1.2; PB, 3.4; and 3-MC, 1.7. The smooth line indicates the protein banding pattern obtained by staining the gels with Coomassie blue. The dotted line indicates the ^{125}I-incorporation profile obtained by fractionating the gel for gamma counting. 40 µg of protein was applied to each gel.

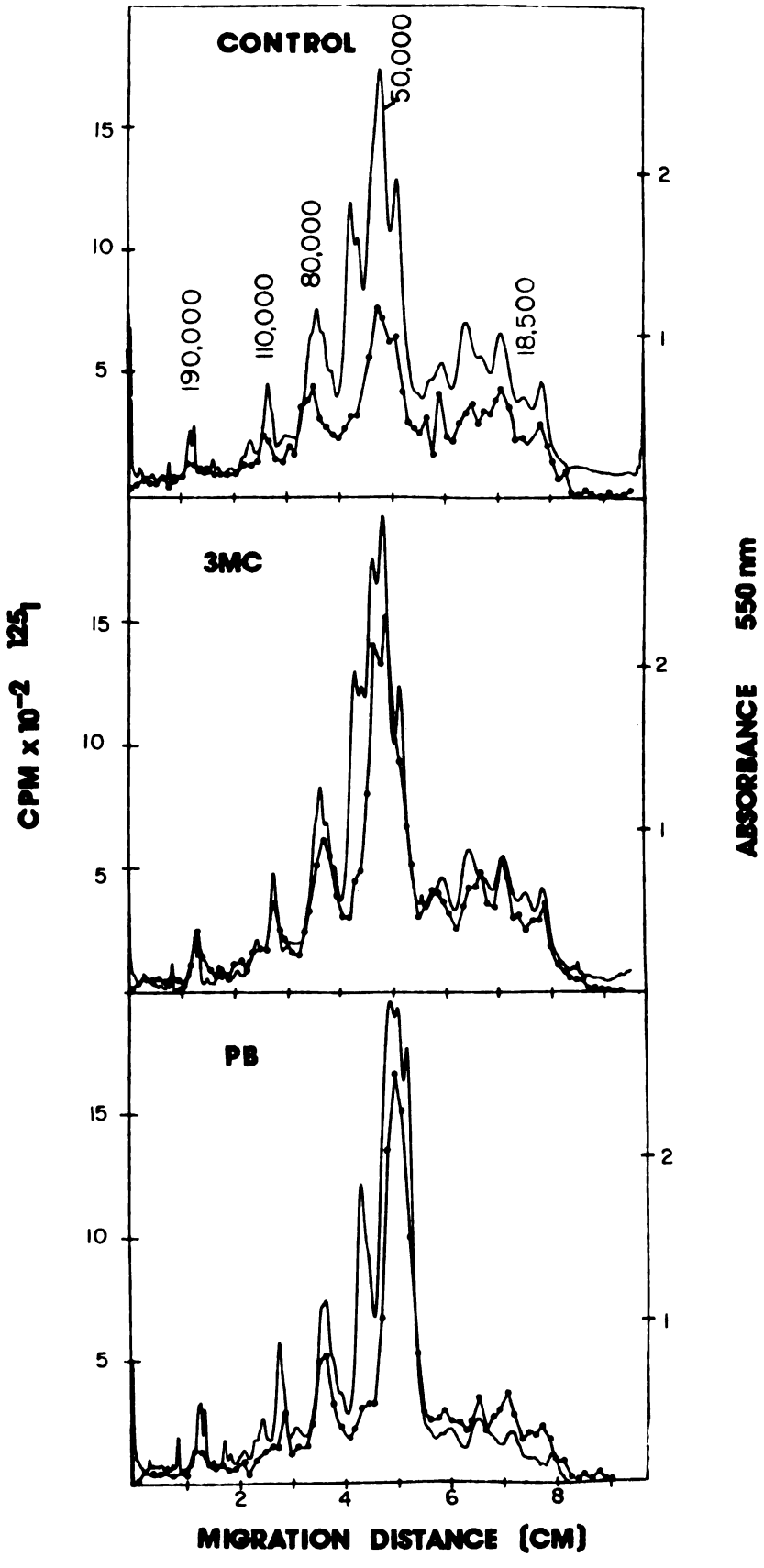
CONTROL
50,000
80,000
110,000
190,000
18,500
3MC
PB
CPM x 10⁻² 125I
ABSORBANCE 550 nm
MIGRATION DISTANCE (CM)

Figure 29

TABLE XI

THE PERCENT OF ^{125}I INCORPORATED INTO MICROSOMAL PROTEINS WHICH APPEARS IN PROTEINS OF 40,000 TO 60,000 DALTONS ON SDS-POLYACRYLAMIDE GELS

Liver microsomes from control, PB-, and 3-MC-pretreated rats were ^{125}I-labeled and electrophoresed on polyacrylamide gels in the presence of 1% sodium dodecylsulfate. After staining the gels to determine protein distribution, the gels were fractionated for gamma counting to determine ^{125}I distribution. The percent of the total cpm recovered from the gel which appeared in fractions from the 40,000 to 60,000 molecular weight region of the gel was then calculated. The values reported are the average and standard deviations from 4 experiments.

Type of microsome	%
Control	36.4 $\pm$ 2.3
PB-pretreated	50.5 $\pm$ 0.5
3-MC-pretreated	43.3 $\pm$ 1.1

proteins in this region besides the 45,000 dalton hemoprotein and the induction of one or all of these proteins may be responsible for the increased ^{125}I-incorporation. Thus while it is likely that the 53,000 dalton hemoprotein induced by 3-MC-pretreatment is located on the exterior of the membrane, more definitive studies will have to be carried out to determine the position of the 45,000 dalton hemoprotein induced by PB.

Discussion

While previous investigations have suggested that different spectral and catalytic forms of cytochrome P_{450} might be present in rat liver microsomes (73,74,85-89,151-156), the studies reported in this chapter, help elucidate the mechanism by which this multiplicity is manifested at the molecular level. Thus it appears that multiple cytochrome P_{450} hemoproteins are present in rat liver microsomes. Three lines of evidence have been postulated which argue for this conclusion: (1) compounds which are known to induce different spectral and catalytic forms of this cytochrome in microsomes were observed, by SDS-gel electrophoresis, to induce different major microsomal protein constituents having molecular weights similar to those suggested for cytochrome P_{450}; (2) the protein constituents induced by these compounds co-purify with cytochrome P_{450} fractions isolated from the induced microsomes; and (3) the induced constituents appear to be hemoproteins since they can be stained with benzidine and H_2O_2 after separation by SDS-gel electrophoresis. Based upon this type of analysis, three cytochrome P_{450} hemoproteins have been observed in the liver microsomes isolated from control rats and these have

apparent molecular weights on SDS-gels of 53,000, 50,000, and 45,000 daltons. The 53,000 dalton hemoprotein is induced by 3-MC-pretreatment of rats while the 45,000 dalton hemoprotein is induced by PB-pretreatment. In actuality, the number three should not be rigorously stressed since it is certainly possible that more cytochrome P_{450} hemoproteins might exist which cannot be resolved by the techniques at hand. In fact, since reporting these results for rat liver microsomes, others (164) have used the techniques of SDS-polyacrylamide gel electrophoresis and benzidine staining, as described in this chapter, to examine rabbit liver microsomes and have concluded that four cytochrome P_{450} hemoproteins are present in these microsomes. In any case, the discovery of multiple cytochrome P_{450} hemoproteins in rat liver endoplasmic reticulum is important not only for our understanding of the mechanism by which multiple mixed-function oxidase activities are attained but it also has important implications for investigators who have been trying to isolate this cytochrome from microsomes. Thus, in isolations, one must be concerned not only with separating the cytochrome from other microsomal proteins but also in separating the different cytochromes from each other.

The obvious key to the discovery of multiple cytochrome P_{450} hemoproteins, reported herein, was their ability to be separated by SDS-polyacrylamide gel electrophoresis. Yet these studies were not the first in which this technique was used to examine the protein constituents of the liver microsomes from control (60,61,67,114,115, 165), and PB- (61,67) or 3-MC-pretreated rats (61). In previous studies which used this technique, however, the proteins were not well resolved and, in some cases, this led investigators to propose that

microsomes contained one main protein component of molecular weight 50,000 daltons (114). In several studies this protein was tentatively identified as cytochrome P_{450} (61,67), and because it appeared to migrate as a single band on SDS-gels, the possibility that multiple cytochrome P_{450} hemoproteins might exist in microsomes was probably not even considered. It is not clear why, in these other studies, more proteins were not resolved in the 50,000 molecular weight region of the SDS-gels. Most of these studies were conducted using 0.1% SDS in the polyacrylamide gels and it was observed in the studies reported in this chapter (Figure 28) that at this lower detergent concentration, the protein components which migrate in the 50,000 molecular weight range on the gels are not as well resolved as they are on gels run using a higher detergent concentration (1% SDS). The cause for this difference is at present unknown. In previous studies, other factors such as the type of buffer, gel length, amount of sample applied to a gel, and the type of staining procedure used also differed from the gel system employed in this chapter. No rigorous comparison of all these parameters has been made. More recently, others have confirmed the finding that multiple protein bands can be resolved in the 50,000 molecular weight region of SDS-gels run on rat liver microsomes (164,166).

Since the three microsomal hemoproteins, tentatively identified as different forms of cytochrome P_{450}, would be thought to have analogous functions, although different substrate specificities, it would be expected that there might be some homology within the structure of these proteins. Also, since the three hemoproteins have different migratory properties on SDS-polyacrylamide gels, it might be

expected that the properties which caused their different migrations on the gels might also be related to the differences in substrate specificities these hemoproteins are postulated to have. If the migration properties of these hemoproteins on SDS-gels are in fact related to their molecular weight, as current theory on SDS-polyacrylamide gel electrophoresis suggests (108), the three proteins appear to differ in size by between 25 to 70 amino acids. This difference in size might be involved in slightly altering the tertiary structure of these hemoproteins resulting in different spatial conformations at the active site of the hemoproteins. Alternatively, it might be that these extra amino acids play a role in the positioning of these hemoproteins within the lipids of the microsomal membrane. In turn, this positioning might influence the type of lipophilic substrate hydroxylated by a certain hemoprotein. Another possibility is that the migration position of these hemoproteins is not influenced as much by size as it is by other properties of the proteins. Thus, while in theory, all proteins are supposed to bind SDS in proportion to their modecular weight, recent studies have indicated that other properties can also influence the amount of SDS bound and hence the migration of membrane-associated proteins in SDS-gels. For example, some membrane proteins appear to contain a high amount of hydrophobic amino acid residues causing them to bind more SDS than they should in proportion to their size. This situation has been reported by Spatz and Strittmatter (33) for the microsomal protein, NADH-cytochrome b_5 reductase. If such proteins do bind a proportionately higher amount of SDS, they would migrate farther into gels during electrophoresis and appear to have a molecular weight lower than is real. Therefore,

it is possible that the differences in the migration properties of the hemoproteins may be related more to a slight difference in the ratio of hydrophobic to hydrophilic amino acid residues in the protein rather than to a large difference in molecular weight. It has also been shown that some glycoproteins do not bind SDS in proportion to their size because carbohydrate components interfere with the binding of the detergent (132). Thus many glycoproteins migrate anomalously on SDS-gels. Perhaps the microsomal cytochrome P_{450} hemoproteins are glycoproteins which differ in their carbohydrate composition. This in turn then might account for the differences in their migration properties on SDS-gels rather than just the size of the proteins. Indeed this may be the case since it has been shown that an analogous hemoprotein, cytochrome P_{450} (cam) from Pseudomonas putida, does appear to contain carbohydrate residues (Personal communication, Dr. Karl Dus, University of Illinois). The mechanism by which a modification in the ratio of hydrophobic to hydrophilic amino acid residues or carbohydrate content might influence the substrate specificity of the multiple hemoproteins is not known but again could be related to a difference in the tertiary structure of the proteins or their positioning within the microsomal membrane. In any case, it will be interesting when such properties as amino acid composition or carbohydrate content can be compared among the different microsomal hemoproteins.

If the results of the studies conducted in both chapters two and three are combined, the simplest model which can be constructed for liver microsomal mixed-function oxidase electron transport would consist of a single type of NADPH-cytochrome c reductase enzyme which transfers electrons from NADPH to three populations of cytochrome

P_{450} which have different substrate specificites. Since the reductase appears to be a minor component of microsomal proteins (Figure 15, chapter two) while the cytochrome P_{450} hemoproteins are major components, it would appear that a single reductase molecule is responsible for the reduction of several cytochrome P_{450}'s during microsomal hydroxylations. Depending upon the source of the liver microsomes (i.e., from control rats or PB- or 3-MC-pretreated rats), the relative amount of the three populations of cytochrome P_{450} would vary. This in turn would account for the different efficiencies these microsomes have in hydroxylating various substrates for the mixed-function oxidases.

Yet the electron transport system may be more complex than this model suggests. For example, from the SDS-gel electrophoresis patterns depicted in Figures 19, 20, and 25, it seems as if PB-pretreatment of rats may induce other microsomal proteins besides the 45,000 dalton hemoprotein thought to be a cytochrome P_{450} hemoprotein. Furthermore, as can be seen in Figures 22 and 23, these proteins co-purify with the cytochrome P_{450} fraction isolated from PB-pretreated rats. Therefore, it may be that in the microsomal membrane, these proteins too are associated with the mixed-function oxidase electron transport chain. Indeed, Autor et al. (167) have provided evidence that in partially purified preparations of cytochrome P_{450} similar to those isolated in this study, the major protein constituents present in the fraction are part of a 350,000 dalton protein complex. It is not known, however, if this is the result of the non-specific aggregation of these membrane components (not an uncommon phenomenon with hydrophobic membrane proteins) or if this is actually because these proteins are associated within the membrane. It will definitely be

interesting to see if these proteins which are induced by PB-pretreatment, but do not appear to be hemoproteins, are associated with the microsomal mixed-function oxidase system and, if so, what role they have in microsomal hydroxylations.

From recent studies, evidence is beginning to accumulate which suggests that other mixed-function oxidase systems may also have multiple forms of cytochrome P_{450}. Thus, on the basis of spectral and substrate specificity studies, the hydroxylation system present in adrenal mitochondria appears to contain a heterogeneous population of cytochrome P_{450} species (168,169). Also, Appleby and Daniel (170) have reported that the soluble mixed-function oxidase system present in the bacteria, Rhizobium japonicum appears to contain spectrally distinct forms of cytochrome P_{450}. These forms were separated from one another by chromatographing the bacterial cellular sap on DEAE-cellulose. It would be interesting to use the technique of SDS-gel electrophoresis in combination with benzidine-staining to determine if the multiple forms of cytochrome P_{450} observed in these systems also result from multiple hemoproteins.

The experiments, reported in this chapter, in which lactoperoxidase-catalyzed protein iodination was combined with SDS-polyacrylamide gel electrophoresis to determine the amount of incorporation of ^{125}I into the 50,000 molecular weight protein constituents of the liver microsomes from control and PB- or 3-MC-pretreated rats suggest that the cytochrome P_{450} hemoproteins may be located on the exterior of the microsomal membrane. Actually, this is not illogical because NADPH-cytochrome c reductase, which transfers electrons from NADPH to the cytochromes, has a similar location (29). If the

cytochrome P_{450} hemoproteins are located on the exterior of the microsomal membrane, it would have some interesting implications for the mechanism of incorporation of these hemoproteins into this membrane during the induction process. Dehlinger and Schimke (61,165) have proposed a theory which suggests that microsomal proteins are synthesized and degraded in the same manner as are cytoplasmic constituents. Thus they speculate that membrane proteins may actually be in a state of equilibrium between associated (membrane-bound) and dissociated (cytoplasmic) states. They have shown that pretreatment of rats with PB and 3-MC results in the specific increase in the synthesis of 50,000 molecular weight microsomal constituents they tentatively identified as cytochrome P_{450}. If their proposal is correct, the cytochromes would be incorporated into a pre-existing membrane because their increased synthesis would have resulted in a larger cytoplasmic pool of these proteins and a shift in the association-dissociation equilibrium to more membrane-bound protein. But the mechanism by which these proteins might then be incorporated into a pre-existing membrane is not clear. Since the studies presented in this chapter suggest that these hemoproteins may be located in the exterior of the microsomal membrane, it may be that binding sites for these proteins are present on the outside of the membrane. Such sites would be analogous to those already found on the exterior of the microsomal membrane for cytochrome b_5 (35). Strittmatter and co-workers have shown that the binding sites for this protein do not appear to be saturated in isolated microsomes, suggesting that the concentration of these proteins in the membrane may be dependent upon the amount available in the cytoplasm for binding. It may be that if external binding sites exist for the

cytochrome P_{450} hemoproteins, these too are not saturated in control microsomes. In that case, the mechanism of insertion of these hemoproteins into the membrane would simply be dependent upon the increase in the cytoplasmic level of these proteins which would follow their increased synthesis. Clearly this hypothesis cannot be substantiated until the various cytochrome P_{450} hemoproteins have been isolated such that the characteristics of their binding to the microsomal membranes from control and PB- or 3-MC-pretreated rats can be studied.

The major finding from the studies presented in this chapter is that multiple cytochrome P_{450} hemoproteins appear to be present in rat liver microsomes. The association of the hemoproteins observed in these studies and cytochrome P_{450} has not been conclusively proven, however. Such proof will require that these hemoproteins be isolated so that they can be spectrally characterized and shown to catalyze specific microsomal hydroxylation reactions. This will not be an easy task because it will require the separate isolation of three proteins having extremely similar spectral and catalytic properties. The experiments presented in the next chapter of this thesis, however, represent a beginning step toward solving this problem.

CHAPTER FOUR

PREPARATION OF ANTIBODY TO THE CYTOCHROME P_{450} HEMOPROTEIN INDUCED IN LIVER MICROSOMES BY PRETREATMENT OF RATS WITH PHENOBARBITAL

Abstract

The 45,000 dalton hemoprotein, induced in rat liver microsomes by pretreatment of animals with PB, is resistant to proteolysis by trypsin and this characteristic was used to purify it from the other major microsomal proteins which appear, by SDS-polyacrylamide gel electrophoresis, to have similar molecular weights. In the procedure used, a sodium cholate-solubilized preparation of cytochrome P_{450} from the liver microsomes of PB-pretreated rats was treated with trypsin and then purified from proteolytic degradation products by Sephadex G-100 column chromatography. The hemoprotein thus isolated appeared, on the basis of SDS-polyacrylamide gel electrophoresis, to be homogeneous, and gave a cytochrome P_{420} spectrum. Antibody was prepared to this hemoprotein and immunoprecipitation studies were conducted using detergent-solubilized partially purified cytochrome P_{450} preparations from control and PB- or 3-MC-pretreated rats. On the basis of these studies, the antibody appeared to interact specifically with the 45,000 molecular weight hemoprotein and not the other

proteins also present in these cytochrome P_{450} preparations. Since this antibody appears to be specific it may be an important tool in studying the microsomal hydroxylation reactions catalyzed by the 45,000 dalton hemoprotein and in studying the orientation of this protein in the microsomal membrane.

Introduction

Rat liver microsomes appear to contain three hemoproteins, which, in 0.1% SDS-polyacrylamide gels, have apparent molecular weights of 53,000, 50,000, and 45,000 daltons. For the remainder of this chapter these will be referred to as hemoproteins 1, 2, and 3, respectively. These hemoproteins were observed by staining gels with benzidine and H_2O_2. Since pretreatment of rats with PB and 3-MC caused their preferential induction, it was suggested that these three proteins may actually represent multiple forms of cytochrome P_{450}. Conclusive proof of this hypothesis, however, will require that these hemoproteins be identified and shown to catalyze specific microsomal hydroxylations.

One approach to this problem would be to prepare antibodies to the three hemoproteins. Such antibodies might be specific inhibitors of the reactions catalyzed by the three hemoproteins and therefore could be used to identify the hydroxylation reactions catalyzed by each hemoprotein. The great advantage of this approach is that the hemoproteins do not have to be isolated in a catalytically active form in order for antibody to be prepared against them. Thus much harsher conditions can be used in the isolations than could be normally employed. One problem which might develop in the use of this

immunochemical approach, however, is that the antibodies prepared to these hemoproteins may cross-react and selective inhibition may not be attainable. In the studies presented in this chapter, a procedure was developed which could be used to isolate hemoprotein 3, which is induced in rat liver microsomes by PB-pretreatment. Antibody directed against this hemoprotein was then prepared and immunoprecipitation studies were carried out to test the cross-reactivity of this antibody with the other 50,000 molecular weight hemoproteins also present in microsomes. These studies suggest that the antibody prepared to hemoprotein 3 is specific for this protein and therefore may be an important tool in identifying the hydroxylation reactions it catalyzes. Also, antibodies directed against specific membrane proteins are becoming important probes for studying the orientation of the proteins within membranes (171) and, in the future, it may be possible to use antibody to hemoprotein 3 for this type of study.

Materials and Methods

Materials

Immature male Sprague-Dawley rats weighing between 75-100 g and mature male rats weighing 225-250 g were obtained from the Spartan Research Animals, Inc., Haslett, Michigan. Male New Zealand rabbits (6-8 lbs) were obtained from the Center for Laboratory Animal Resources, Michigan State University.

Freund's complete adjuvant was obtained from Difco Laboratories, Detroit, Michigan.

The sources for other materials were listed in chapters one, two, and three.

Drug Pretreatment of Animals

3-MC-pretreatment of rats was carried out on immature male 75-100 g rats as described in chapter two. PB-pretreatment was carried out on mature male rats as described in chapter one.

Isolation of Hemoprotein 3 from the Liver Microsomes of a PB-Pretreated Rat

Unless otherwise indicated, all procedures were carried out at 4°C. Liver microsomes were isolated from PB-pretreated rats as described in chapter one. These microsomes (800-1000 mg) were washed with 1.15% KCl containing 10 mM EDTA, pH 7.5 and solubilized with sodium cholate as described in chapter three for the partial purification of cytochrome P_{450}. After centrifugation at 105,000 x g for 90 minutes, the cholate-solubilized microsomal proteins were then fractionated with ammonium sulfate. The fraction precipitating between 40 to 50% saturation was suspended in approximately 13 ml of 0.05 M Tris-HCl, pH 7.5 containing 0.1 mM EDTA and 0.1 mM dithiothreitol, centrifuged at 160,000 x g for 90 minutes and dialyzed overnight against 2 l. of the same buffer. This preparation was then digested with trypsin for 6 hours. Typically 5 ml (75 mg) was digested at one time. This digestion was carried out at a ratio of 1 mg of trypsin per 15 mg of partially purified cytochrome P_{450}. During proteolysis cytochrome P_{450} was converted to its P_{420} spectral form. The proteolytic reaction was terminated by adding soybean trypsin inhibitor (2 mg trypsin inhibitor:1 mg trypsin) and the sample was centrifuged at 35,000 x g for 20 minutes. The supernatant was applied to a Sephadex G-100 column (30 cm x 3 cm) to remove proteolytic degradation fragments, trypsin, and trypsin inhibitor. The column was eluted with

0.05 M Tris-HCl, pH 7.5 containing 0.1 mM EDTA and 0.1 mM dithiothreitol. The column fractions were assayed for cytochrome P_{420} (62), NADPH-cytochrome c reductase (98), cytochrome b_5 (46), and protein (103). The fractions obtained from this column which had the highest specific activity of cytochrome P_{420} contained hemoprotein 3 and were used for antibody production.

For some experiments the cytochrome P_{420} fractions from the Sephadex G-100 column were concentrated to approximately 8 mg/ml and then solubilized in 0.01 M Tris-HCl, pH 8.0 (at 25°C) containing 2% SDS, 7% sucrose, 1 mM EDTA, and 40 mM dithiothreitol. The mixture was boiled for 15 minutes, cooled, and then applied to a Sephadex G-200 column (30 cm x 3 cm) which was eluted with 0.04 M Tris-HCl, pH 7.5 (at 25°C) containing 0.02 M sodium acetate, 1% SDS, and 2 mM EDTA. The column fractions were assayed for protein by following the absorbance at 280 nm.

Immunological Techniques

Antibody was prepared by immunizing an adult male rabbit with three injections of the hemoprotein 3 preparation (3 mg protein/injection). This antigen was administered in 2 ml of 50% Freund's complete adjuvant at one week intervals. Each weekly administration consisted of multiple intradermal injections of approximately 0.1 ml into the abdomen. Blood was collected from an ear vein 10 days after the final injection and serum was separated from the whole blood by allowing the blood to clot at room temperature for 3 to 4 hours. The clotted blood was then centrifuged at 10,000 x g for 20 minutes at room temperature and the whole sera was removed by decantation. The

IgG fraction from both immune and pre-immune serum was then prepared by ammonium sulfate fractionation and DEAE-column chromatography (172). The IgG fraction was used in all experiments described in this chapter.

Ouchterlony double diffusion analysis (148) was performed as described in chapter two.

Iodination of Partially Purified Cytochrome P_{450} Fractions from Control, PB-, and 3-MC-Pretreated Rats

Partially purified cytochrome P_{450} fractions were prepared from liver microsomes as described in chapter three. For some experiments the proteins present in these cytochrome fractions were iodinated using lactoperoxidase, $^{125}I^-$, and H_2O_2. The cytochrome fractions were diluted to a concentration of 12 nmole cytochrome P_{450}/ml (2-4 mg of protein/ml) in 0.0025 M potasssium phosphate buffer, pH 7.7, 10% glycerol, 0.05 mM dithiothreitol, 0.05 mM EDTA, 0.05% sodium cholate, 5×10^{-6} M KI (containing 200 μC $^{125}I^-$/ml), 2×10^{-6} M lactoperoxidase, and 0.005% BHT. Iodination was carried out at 25°C by the addition of aliquots of 85 nmoles H_2O_2/ml reaction at 1 minute intervals. Six additions were made. The samples were then chromatographed on a small Biogel P-10 column (prepared in a Pasteur pipet) to remove unreacted $^{125}I^-$ and H_2O_2. Lactoperoxidase and H_2O_2 concentrations were determined spectrophotometrically as described in chapter one.

Immunoprecipitation of Hemoprotein 3 from Detergent-Solubilized Partially Purified Cytochrome P_{450} Fractions from Control, PB-, and 3-MC-Pretreated Rats

Studies to determine which protein constituent(s) of partially purified cytochrome P_{450} preparations from control and PB- or 3-MC-pretreated rats formed an immunoprecipitate with antibody to the hemoprotein 3 preparation were conducted by suspending ^{125}I-labeled cytochrome P_{450} preparations to a concentration of 0.5 mg/ml in 0.01 M sodium phosphate, pH 8.0 containing 1% sodium deoxycholate and 10 mM EDTA. IgG, also suspended in this same buffer was added in an 80:1 protein of IgG to protein of P_{450} ratio. Immunoprecipitation was allowed to proceed at 4°C overnight. The immunoprecipitates formed were collected by centrifugation at 3,000 x g for 10 minutes and washed with 0.01 M sodium phosphate buffer, pH 8.0. The immunoprecipitate was then suspended in 0.01 M Tris-HCl, pH 8.0 (at 25°C) containing 1% SDS, 7% sucrose, 1 mM EDTA, and 10 µg/ml pyronin B tracking dye before heating at 100°C for 1 minute prior to 1% SDS-gel electrophoresis.

Immunoprecipitation experiments to assay for the presence of cytochrome P_{420} in the precipitate were conducted using a procedure similar to that described above. In this case, however, the immunoprecipitation was carried out in buffer containing 0.25% sodium deoxycholate. After washing, the precipitate was then resuspended in 0.3 M sodium phosphate buffer, pH 7.5 for reduced, CO-difference spectroscopy (62) or in 1% SDS-buffer for 1% SDS-polyacrylamide gel electrophoresis.

SDS-Polyacrylamide Gel Electrophoresis

1% SDS-polyacrylamide gel electrophoresis was performed as described in chapter one. In some cases, dithiothreitol was not included in the samples for electrophoresis, however, This electrophoresis technique was modified, as described in chapter three when gels were stained with benzidine and H_2O_2 to visualize hemoproteins. For benzidine-staining, the cytochrome P_{450}-enriched fraction used for trypsinization and the hemoprotein 3 fractions from the Sephadex G-100 column were each suspended to a protein concentration of 3 mg/ml in 0.01 M Tris-HCl, pH 8.0 (at 25°C) containing 0.5% SDS, 7% sucrose, 1 mM EDTA, and 10 µg/ml pyronin B tracking dye. They were then immediately applied to 0.1% SDS-gels and subjected to the electrophoresis and benzidine-staining procedure as described in chapter three.

Results

Isolation of Hemoprotein 3

The procedure which was developed for the isolation of hemoprotein 3 had its origins in various experiments which were conducted to find techniques which could be used to separate the three microsomal hemoproteins thought to be multiple forms of cytochrome P_{450}. During such exploratory experiments, one curious property of hemoprotein 3 was discovered in that it appeared to be resistant to proteolysis by trypsin. Other microsomal proteins which migrate with approximate molecular weights of 50,000 on SDS-gels were readily degraded, however. Therefore it seemed that this was a convenient property to use in the isolation of this hemoprotein from the other major microsomal constituents.

For the isolation, PB-microsomes were chosen because they are greatly enriched in hemoprotein 3 and appear to have a very low concentration of the other hemoproteins (Figure 26, chapter three). The complete isolation procedure is described in the Materials and Methods section and essentially consisted of treating a sodium cholate-solubilized cytochrome P_{450}-enriched fraction from PB-microsomes with trypsin for 6 hours (during proteolysis cytochrome P_{450} was converted to its P_{420} form), adding soybean trypsin inhibitor to stop the digest, and chromatographing the proteolysis mixture on Sephadex G-100 to remove trypsin, trypsin inhibitor, and proteolytic degradation fragments. An elution profile from the column is shown in Figure 30. Cytochrome P_{420} appeared to elute in the void volume of this column. This was also found to be true when chromatography was performed on Sepahdex G-200 and on Sepharose 4B, suggesting that the cytochrome P_{420} was part of a high molecular weight aggregate. It is not uncommon for hydrophobic membrane proteins to form such aggregates when they are removed from membranes (132). The enzymes, NADPH-cytochrome c reductase and cytochrome b_5, which were also found in the cytochrome P_{450}-enriched fraction used for proteolysis, were separated from cytochrome P_{420} during the Sephadex G-100 column chromatography. These proteins eluted in positions which suggested they had been proteolytically degraded by trypsin (29,58,59,98).

The fractions from the Sephadex G-100 column which contained the highest specific activity of cytochrome P_{420} were analyzed by 1% SDS-polyacrylamide gel electrophoresis. Such analyses indicated that these fractions consisted of a single protein which had the same migratory properties during SDS-gel electrophoresis as the 45,000

Figure 30. SEPHADEX G-100 COLUMN CHROMATOGRAPHY OF A TRYPSIN-TREATED CYTOCHROME P_{450}-ENRICHED FRACTION FROM THE LIVER MICROSOMES OF A PB-PRETREATED RAT

The experimental procedures have been described in the Materials and Methods. The fraction volumes were 2 ml. The void volume for the column, determined by chromatography of blue dextran, corresponded to fractions 35-40.

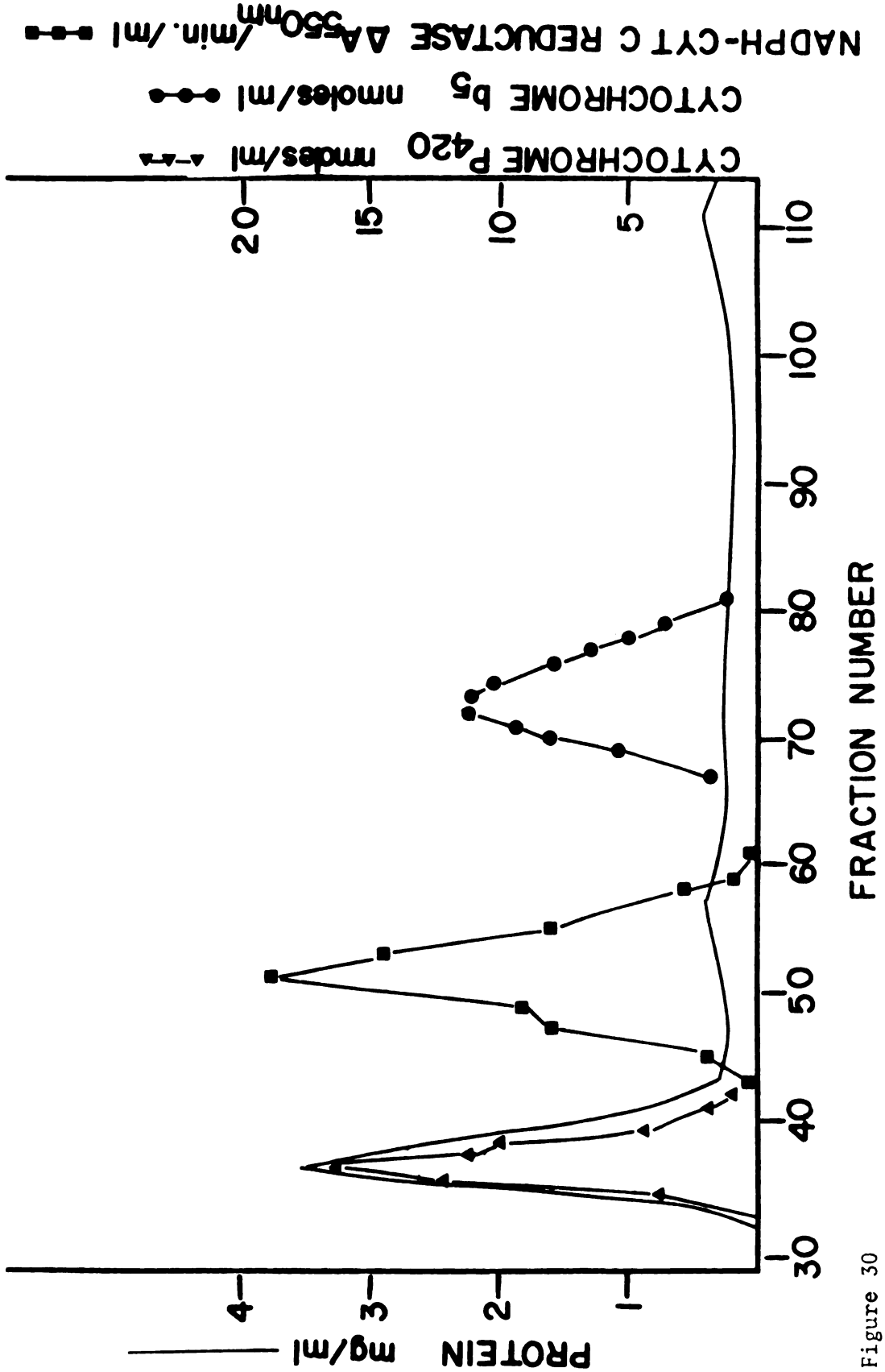
PROTEIN mg/ml
CYTOCHROME P420 nmoles/ml
CYTOCHROME b5 nmoles/ml
NADPH-CYT C REDUCTASE ΔA550nm/min./ml
FRACTION NUMBER
30
40
50
60
70
80
90
100
110
1
2
3
4
5
10
15
20

Figure 30

dalton component originally present in the cytochrome P_{450}-enriched fraction used for proteolysis (Figure 31). Because this protein was prepared by proteolysis, it may have been partially degraded and could not be directly correlated with this protein component of the cytochrome P_{450} preparation, however. Electrophoresis of the cytochrome P_{420} fractions from the Sephadex G-100 column on 0.1% SDS-gels for benzidine-staining indicated that the major component of these fractions was a hemoprotein and appeared to migrate in nearly the same position on these gels as hemoprotein 3, the major hemoprotein found in PB-microsomes (Figure 32). Therefore it seemed, on the basis of such analyses, that a method had been found which could be used to isolate hemoprotein 3 from PB-microsomes in a form suitable for antibody production. Furthermore, because this hemoprotein gave a cytochrome P_{420} spectrum when assayed by reduced, CO-difference spectroscopy, it appeared to be an altered form of cytochrome P_{450}.

One incongruency in the isolation procedure, however, resulted from trying to correlate the purity of the hemoprotein with cytochrome P_{420} specific activity data. Thus while it appeared, by SDS-gel electrophoresis, that a pure hemoprotein had been isolated, the maximum specific activities of cytochrome P_{420} attained by this procedure ranged from 5 to 9 nmole/mg protein (20 nmole/mg would be expected for a pure protein). Several explanations can be offered for this, however. One is that during proteolysis the protein is altered in such a fashion that the extinction coefficients normally used for calculating cytochrome P_{420} concentrations are no longer valid. A second is that heme dissociated from the cytochrome P_{450} hemoprotein during the isolation and this, in fact, is probably very true since

Figure 31. 1% SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROTEIN PROFILES OF THE CYTOCHROME P_{450}-ENRICHED FRACTION BEFORE TRYPSIN TREATMENT AND THE CYTOCHROME P_{420} PREPARATION RESULTING FROM TRYPSIN DIGESTION AND SEPHADEX G-100 COLUMN CHROMATOGRAPHY

The upper scan is of the cytochrome P_{450}-enriched preparation used for proteolysis while the lower scan is of the cytochrome P_{420} preparation after Sephadex G-100 column chromatography. The samples were prepared in the presence of 40 mM dithiothreitol. After electrophoresis the gels were stained with Coomassie blue and scanned at 550 nm. 24 µg of protein was applied to the upper gel while 15 µg was applied to the lower.

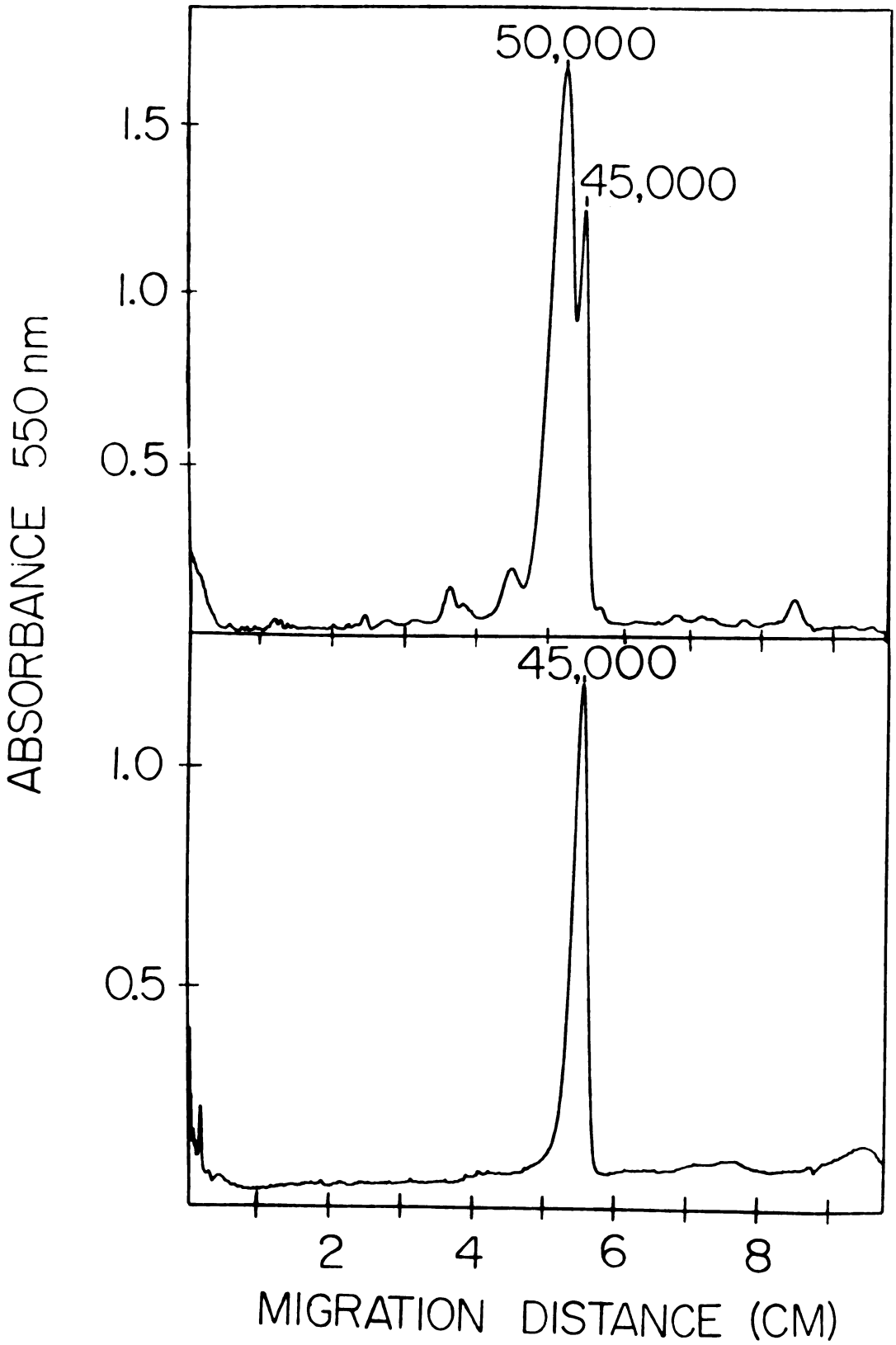
50,000
45,000
1.5
1.0
0.5
ABSORBANCE 550 nm
45,000
1.0
0.5
2
4
6
8
MIGRATION DISTANCE (CM)

Figure 31

Figure 32. 0.1% SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS HEMOPROTEIN PROFILES OF THE CYTOCHROME P_{450}-ENRICHED FRACTION BEFORE TRYPSIN TREATMENT AND THE CYTOCHROME P_{420} PREPARATION RESULTING FROM TRYPSIN DIGESTION AND SEPHADEX G-100 COLUMN CHROMATOGRAPHY

The gel on the left is of the cytochrome P_{450}-enriched fraction before proteolysis while the gel on the right is of the cytochrome P_{420} preparation obtained by column chromatography. The hemoprotein profiles were visualized by staining the gels with benzidine and H_2O_2 as described in the Materials and Methods. 40 µg of each sample was applied to the gels.

Figure 32

heme is known to dissociate from cytochrome P_{420} very easily (162,163). Finally, a third explanation is suggested by the observation that the hemoprotein is eluted from the Sephadex G-100 column in the form of a high molecular weight aggregate. Thus it is possible that this aggregate contains, in addition to hemoprotein 3, small hydrophobic peptides which undoubtedly result when membrane proteins are proteolytically degraded. Such peptides could be of such small molecular weights that they could not be observed on SDS-gels. To further explore this third possibility, the hemoprotein 3 preparation was solubilized in SDS and chromatographed on Sephadex G-200 also in the presence of this detergent. The results of such an experiment are shown in Figure 33. It can be seen that two peaks of material which absorb at 280 nm eluted from the column during this procedure. When the first peak was analzyed by 1% SDS-gel electrophoresis, it contained the 45,000 dalton protein but when the second peak of material was similarly analyzed, no proteins could be detected on the gel. Because the results of this experiment confirmed the possibility that the cytochrome P_{420} preparation might also contain small peptides, in addition to hemoprotein 3, which are not detectable by SDS-gel electrophoresis, initial attempts at preparing antibody to this hemoprotein employed protein which had been further purified by SDS-column chromatography on Sephadex G-200. In this form the protein was not a good antigen, however, and eventually antibody was prepared by injecting the entire hemoprotein 3 aggregate into rabbits. In the following experiments, the antibody formed to this antigen was further characterized.

Figure 33. SDS-SEPHADEX G-200 COLUMN CHROMATOGRAPHY OF THE CYTOCHROME P_{420} PREPARATION

A cytochrome P_{420} preparation obtained by trypsin digest of a cytochrome P_{450}-enriched fraction from PB-microsomes and Sephadex G-100 column chromatography was subsequently solubilized in SDS-buffer and chromatographed on Sephadex G-200 also in the presence of this detergent. The void volume was determined by chromatographing blue dextran on the column and assaying its absorbance at 550 nm. Protein was assayed by its absorbance at 280 nm.

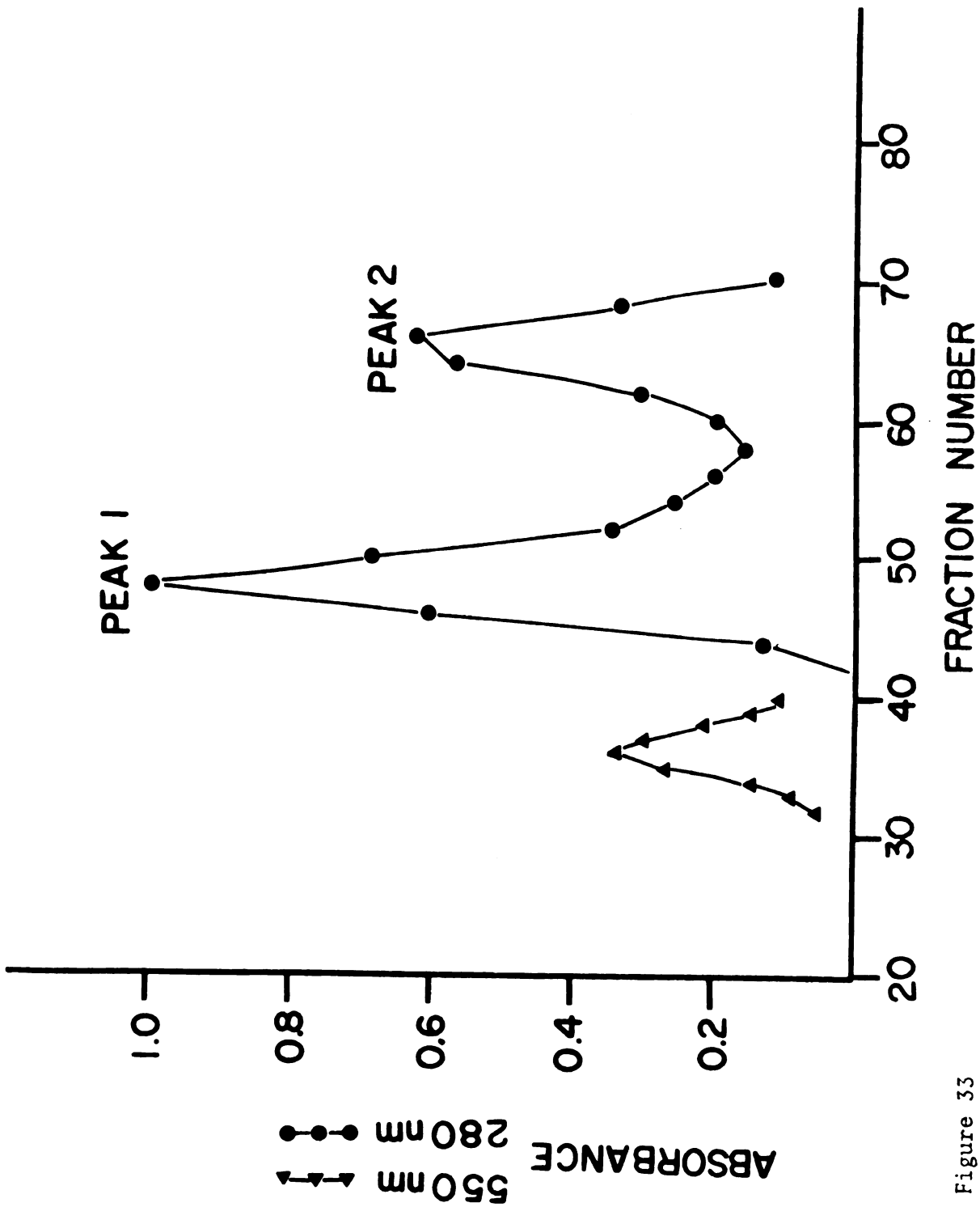
PEAK 1
PEAK 2
1.0
0.8
0.6
0.4
0.2
ABSORBANCE
280nm
550nm
20
30
40
50
60
70
80
FRACTION NUMBER

Figure 33

Ouchterlony Double Diffusion Analysis of the Precipitin Reactions Between the Antibody to Hemoprotein 3, the Hemoprotein 3 Antigen, and Partially Purified Cytochrome P_{450} Preparations from Control and PB- or 3-MC-Pretreated Rats

Immunoprecipitation studies to characterize the antibody produced against hemoprotein 3 were conducted using partially purified cytochrome P_{450} preparations isolated from the liver microsomes of control and PB- or 3-MC-pretreated rats. The isolation procedure of Levin et al. (66) was used, as modified in chapter three. Since the control and 3-MC-cytochrome P_{450} preparations were shown in chapter three to contain all three hemoproteins, immunoprecipitation studies conducted with these preparations were particularly important in assessing the specificity of the antibody for only hemoprotein 3. While Autor et al. (167) have shown that the major components of the P_{450} preparations are associated together in a 350,000 molecular weight protein complex, it was found that these proteins could be solubilized from one another in the presence of sodium deoxycholate. Therefore immunoprecipitation studies were carried out in the presence of this detergent.

Ouchterlony double diffusion analysis (Figure 34) demonstrated that the antibody would form a single precipitin line when reacted against its antigen and each of the three types of detergent-solubilized partially purified cytochrome P_{450} preparations. Therefore antibody had been formed against a single constituent which was present in the antigen and also in the partially purified cytochrome P_{450} fractions prepared from the liver microsomes of control and PB- or 3-MC-pretreated rats. Furthermore, since the precipitin lines

Figure 34. OUCHTERLONY DOUBLE DIFFUSION ANALYSES OF THE PRECIPITIN REACTIONS BETWEEN THE HEMOPROTEIN 3 ANTIBODY, ITS ANTIGEN, AND PARTIALLY PURIFIED CYTOCHROME P_{450} PREPARATIONS FROM THE LIVER MICROSOMES OF CONTROL AND PB- OR 3-MC-PRETREATED RATS

The center well contained 90 µg of IgG prepared from rabbit antiserum, as described in the Materials and Methods. The outer wells, numbered in a clockwise fashion contained the following samples, suspended to a concentration of 1-3 mg/ml in 0.015 M sodium phosphate buffer, pH 7.5 containing 2% sodium deoxycholate: Well 1, 54 µg of hemoprotein antigen; wells 2 and 5, 24 µg of PB P_{450}; well 3, 0.015 M sodium phosphate buffer containing 2% sodium deoxycholate; well 4, 84 µg of control P_{450}; and well 6, 34 µg of 3-MC P_{448}.

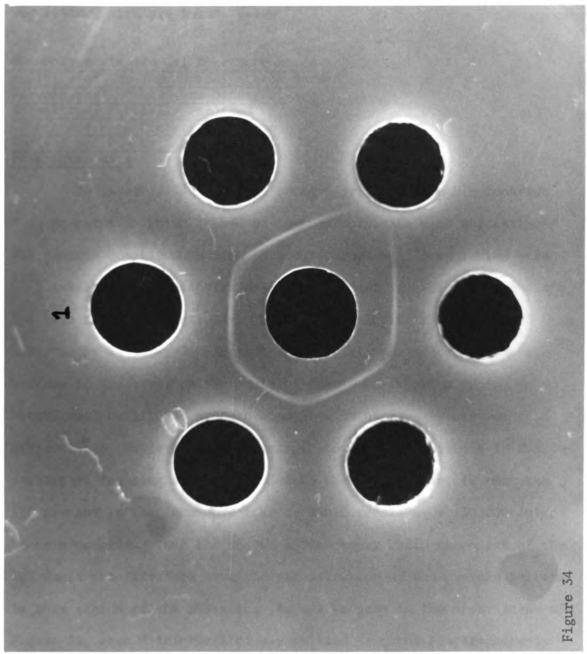

Figure 34

formed with all these samples fused, the component precipitated from each sample were immunologically identical. No immunoprecipitates were formed when the hemoprotein 3 antigen and each cytochrome P_{450} fraction was reacted with pre-immune serum.

SDS-Polyacrylamide Gel Electrophoretic Analysis of the Immunoprecipitates Formed Between the Antibody and Detergent-Solubilized Partially Purified Cytochrome P_{450} Preparations from Control and PB- or 3-MC-Pretreated Rats

To directly identify the protein present in each cytochrome P_{450} preparation which formed an immunoprecipitate with the antibody, the protein constituents from each cytochrome P_{450} preparation were ^{125}I-labeled with lactoperoxidase, solubilized in sodium deoxycholate, and then mixed with antibody. The immunoprecipitates formed were then analyzed by 1% SDS-gel electrophoresis. This gel system was chosen because it appeared, on the basis of Figure 28 (chapter three) to better resolve all the protein constituents of each cytochrome P_{450} preparation. While the immunoprecipitates were solubilized in SDS, no sulfhydryl reducing agent was added to the preparation, so that the IgG present in the immunoprecipitate would remain as a 150,000 dalton protein complex. Thus the 50,000 dalton heavy chain component of the IgG would not interfere with the visualization of the protein pattern in this region of the SDS-gels. As can be seen in the upper scans of Figure 35, when dithiothreitol was omitted from the electrophoresis sample the migratory properties of the polypeptide components of each partially purified cytochrome P_{450} preparation did not change from those previously described in Figure 22 of chapter three. The bottom

Figure 35. A COMPARISON OF THE 1% SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROTEIN PROFILES OF PARTIALLY PURIFIED CYTOCHROME P_{450} PREPARATIONS ISOLATED FROM THE LIVER MICROSOMES OF CONTROL AND PB- OR 3-MC-PRETREATED RATS AND THE IMMUNOPRECIPITATES FORMED BETWEEN EACH CYTOCHROME P_{450} PREPARATION AND THE HEMOPROTEIN 3 ANTIBODY

The upper scans are of partially purified cytochrome P_{450} (448) fractions prepared from the liver microsomes of control, PB- and 3-MC-pretreated rats, as described in chapter three. The lower scans are of the immunoprecipitates formed between these cytochrome P_{450} preparations (after ^{125}I-labeling) and the antibody. Dithiothreitol was not added to the samples prior to electrophoresis. The solid lines are the protein banding patterns visualized by Coomassie blue staining. The dotted line represents the ^{125}I pattern of the immunoprecipitate which was obtained by fractionating the gels for gamma counting.

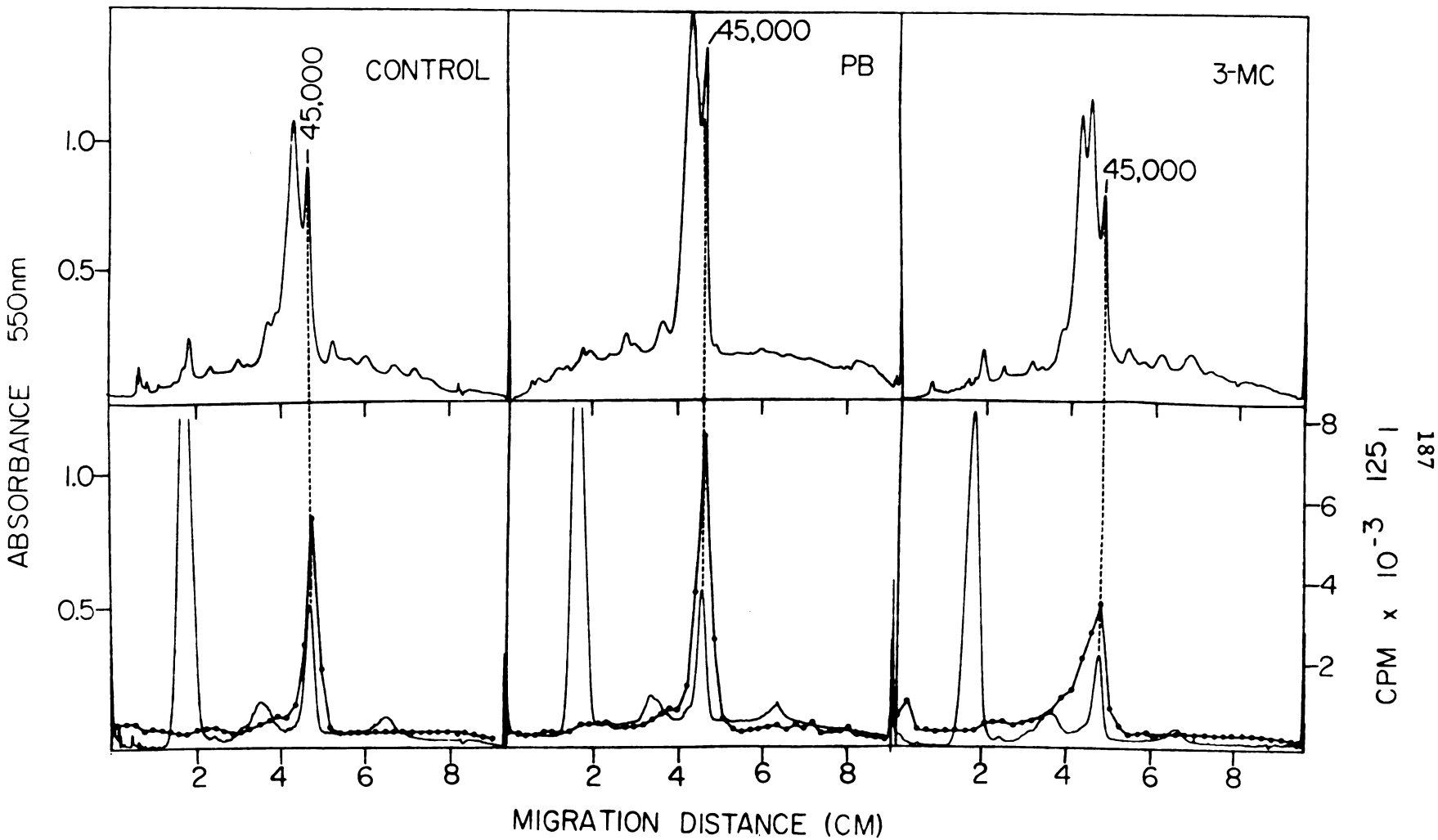
CONTROL
45,000
PB
45,000
3-MC
45,000
ABSORBANCE 550nm
1.0
0.5
1.0
0.5
2
4
6
8
2
4
6
8
2
4
6
8
MIGRATION DISTANCE (CM)
CPM x 10^{-3} ^{125}I
2
4
6
8

Figure 35

scans of Figure 35 are of the immunoprecipitates formed between antibody and the corresponding cytochrome P_{450} preparation. Each immunoprecipitate appeared to contain one protein which was ^{125}I-labeled and therefore was the component of the cytochrome P_{450} preparation immunoprecipitated by the antibody. In each case this protein corresponded to the 45,000 dalton component present in the preparation. That the component immunoprecipitated from each cytochrome fraction has an identical molecular weight is further emphasized in Figure 36 which directly compares the immunoprecipitates obtained from each type of cytochrome P_{450}. Other proteins are also visible in each immunoprecipitate and have molecular weights of 150,000, 75,000, and 25,000. There are probably IgG (150,000) or products of the partial dissociation of this complex in SDS. It therefore appears from these immunoprecipitation studies that the antibody was specific for the 45,000 molecular weight component present in each cytochrome P_{450} preparation. Furthermore, this suggests that the 45,000 dalton hemoprotein antigen used to prepare the antibody was not detectably degraded during the proteolysis procedure used in its purification.

To directly identify the protein immunoprecipitated as a hemoprotein, an immunoprecipitate formed between the antibody to hemoprotein 3 and detergent-solubilized partially purified cytochrome P_{450} isolated from the liver microsomes of PB-pretreated rats was assayed by reduced, CO-difference spectroscopy. This immunoprecipitation experiment was performed in 0.25% sodium deoxycholate because higher detergent concentrations dissociated the heme from the hemoproteins present in this partially purified cytochrome P_{450} preparation. As can be seen from the gel scan presented in Figure 37, at this lower

Figure 36. A COMPARISON OF THE 1% SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROTEIN PROFILES OF THE IMMUNOPRECIPITATES FORMED BETWEEN THE HEMOPROTEIN 3 ANTIBODY AND DETERGENT-SOLUBILIZED PARTIALLY PURIFIED CYTOCHROME P_{450} PREPARATIONS FROM THE LIVER MICROSOMES OF CONTROL, PB-, AND 3-MC-PRETREATED RATS.

Immunoprecipitation and electrophoresis was performed as described in the legend to Figure 35.

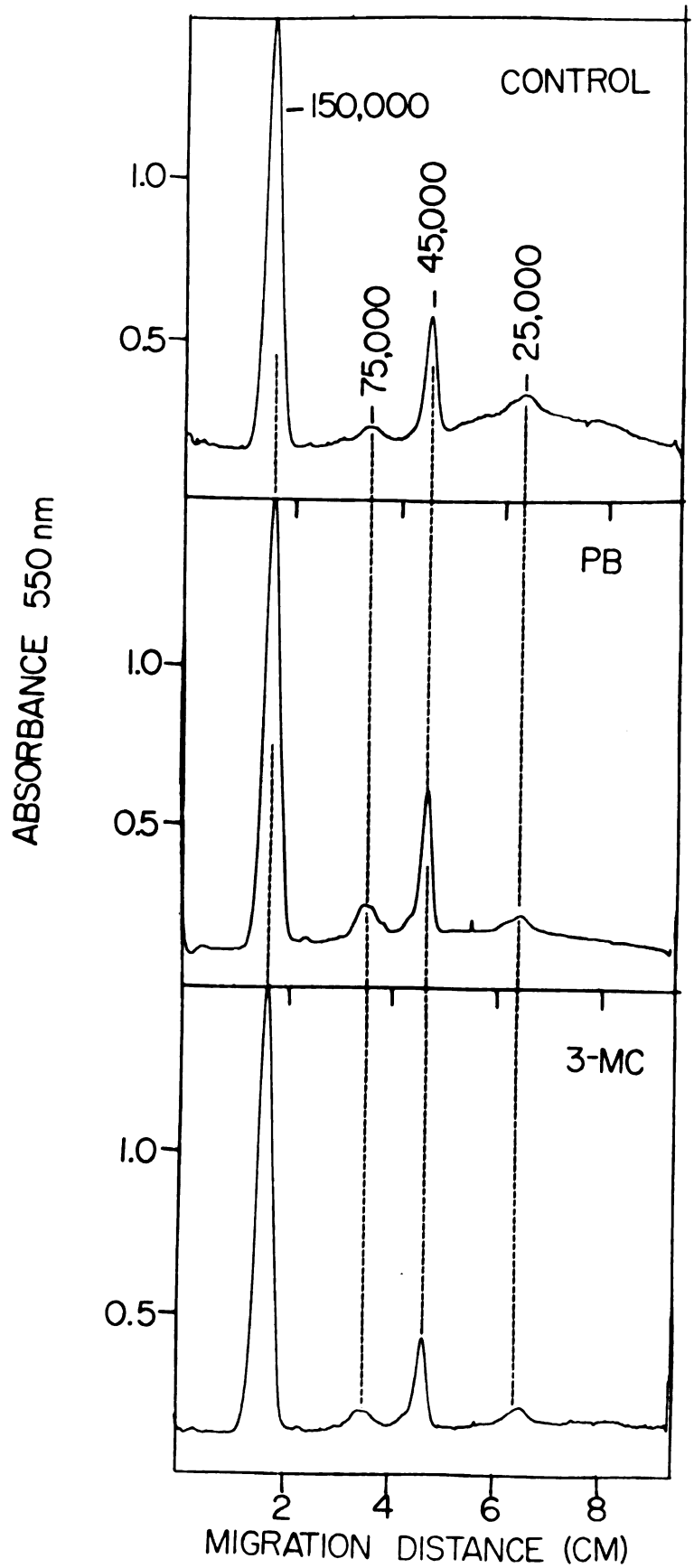
CONTROL
150,000
75,000
45,000
25,000
PB
3-MC
1.0
0.5
ABSORBANCE 550 nm
2
4
6
8
MIGRATION DISTANCE (CM)

Figure 36

Figure 37. SPECTRAL AND ELECTROPHORETIC ANALYSIS OF THE IMMUNOPRECIPITATES FORMED BETWEEN THE HEMOPROTEIN 3 ANTIBODY AND A DETERGENT-SOLUBILIZED PARTIALLY PURIFIED CYTOCHROME P_{450} PREPARATION FROM THE LIVER MICROSOMES OF A PB-PRETREATED RAT

The immunoprecipitate formed between the antibody and a detergent-solubilized partially purified cytochrome P_{450} preparation from PB microsomes was assayed both by 1% SDS-polyacrylamide gel electrophoresis and reduced, CO-difference spectroscopy. For this experiment the immunoprecipitation was carried out in the presence of 0.25% sodium deoxycholate. The precipitates formed were then either resuspended in 0.3 M sodium phosphate, pH 7.5 for spectral assay (inset) or 1% SDS-buffer (without dithiothreitol) for electrophoresis.

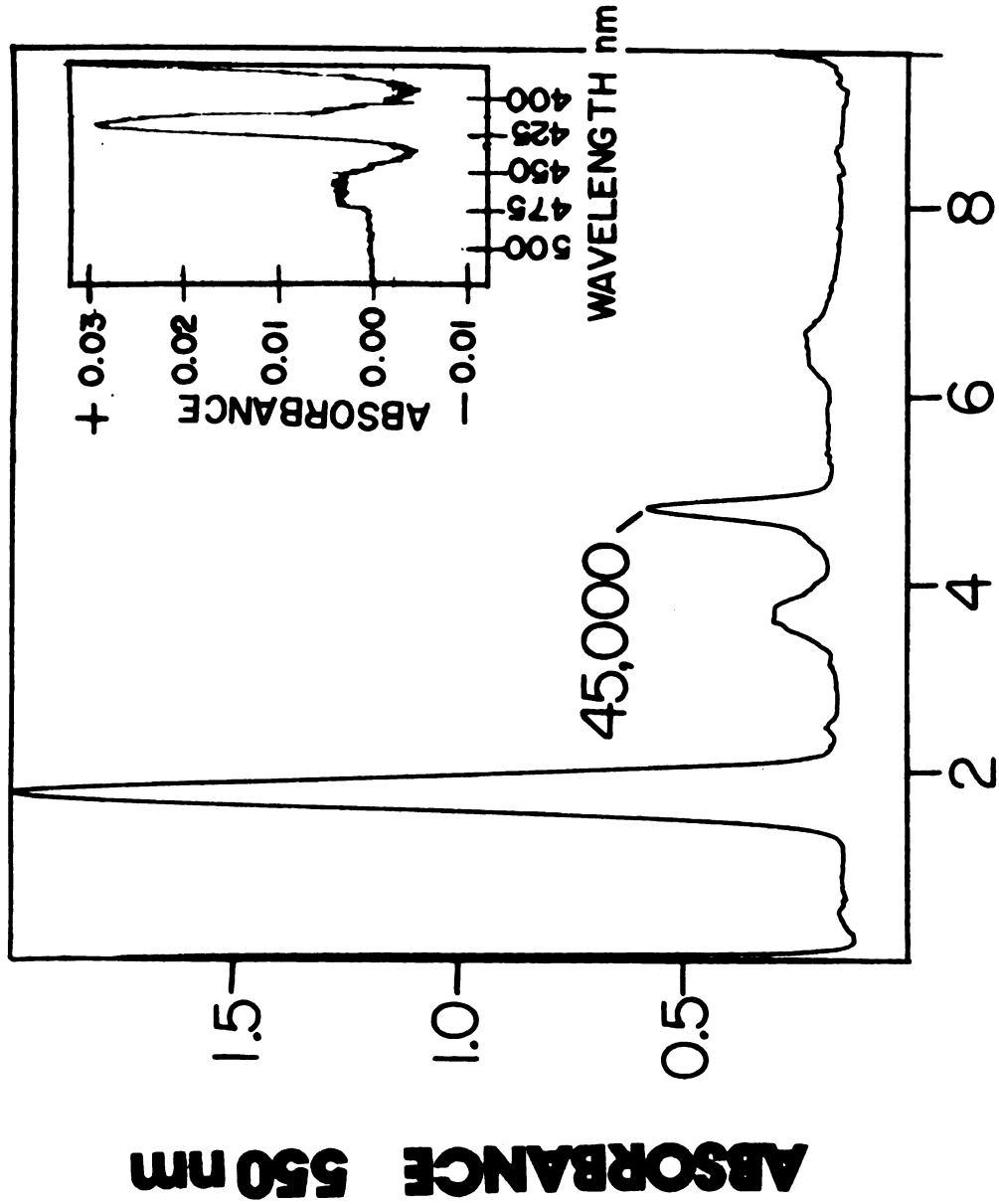
ABSORBANCE 550 nm
1.5
1.0
0.5
45,000
2
4
6
8
MIGRATION DISTANCE CM
WAVELENGTH nm
400
425
450
475
500
ABSORBANCE
+ 0.03
0.02
0.01
0.00
- 0.01

Figure 37

detergent concentration, the 45,000 dalton protein still appeared to be the only component from the partially purified cytochrome P_{450} preparation which was immunoprecipitated by the antibody. Spectral analysis of the immunoprecipitate indicated that it contained a hemoprotein with a spectrum characteristic of cytochrome P_{420} (inset, Figure 37). Others have observed that sodium deoxycholate converts cytochrome P_{450} hemoproteins to their cytochrome P_{420} form (47,60). Therefore, since the immunoprecipitation was carried out in the presence of this detergent, it is not surprising that the spectral form of the hemoprotein precipitated by the antibody would be cytochrome P_{420}. This experiment then confirms that the 45,000 dalton protein immunoprecipitated by the antibody is a cytochrome P_{450} hemoprotein.

Discussion

The studies presented in this chapter demonstrate that hemoprotein 3, the 45,000 dalton hemoprotein observed by electrophoresing rat liver microsomal protein on 0.1% SDS-polyacrylamide gels, is indeed a cytochrome P_{450} hemoprotein. This is the form of cytochrome P_{450} described in chapter three to be induced in rat liver microsomes by pretreatment of animals with PB. Furthermore, through the studies conducted in this chapter it has been demonstrated that this hemoprotein migrates as a protein of identical molecular weight on both 0.1% and 1% SDS-gels. Therefore, this hemoprotein can be identified as protein component 6 in the 1% SDS-polyacrylamide gel protein profiles described in chapter three (Figures 20, 22, and 23).

The identification of hemoprotein 3 as one form of cytochrome P_{450} was based upon the observation that the major portion of this

protein is not degraded by trypsin. Therefore, a form of this hemoprotein, suitable for antibody production, could be isolated by using trypsin to proteolytically degrade the other major protein constituents found in a cytochrome P_{450}-enriched fraction isolated from the microsomes of PB-pretreated rats. The polypeptide degradation fragments resulting from the proteolysis of these proteins were then removed from this hemoprotein by Sephadex G-100 column chromatography. As previously discussed, the hemoprotein thus prepared appears to be part of a high molecular weight protein aggregate. Since the molecular weight of the hemoprotein components of this aggregate appeared, by SDS-polyacrylamide gel electrophoresis, to be identical to the molecular weight of the "native" hemoprotein, obtained by immunoprecipitation studies, it seems that trypsin does not noticably degrade this hemoprotein. Other studies, such as N-terminal analysis have not been performed on the form of the hemoprotein isolated by proteolysis and therefore it is not known if there is, in fact, some slight degradation of this hemoprotein by the protease. The observation that during proteolysis the spectral form of the cytochrome was changed from its P_{450}-form to a P_{420}-form, however, suggests that some type of alteration may be occurring. In any case, since the protein was only to be used for antibody production, this was not of great concern.

The immunoprecipitation analyses which were conducted in this chapter to test the specificity of the antibody prepared to the hemoprotein 3 antigen were performed using partially purified cytochrome P_{450} fractions isolated from rat liver microsomes. These fractions were used instead of whole microsomal protein because of problems which were encountered in trying to find the correct balance between a

detergent concentration which would completely solubilize hemoprotein 3 from microsomes and still not interfere with the antigen-antibody interaction. Obviously if the hemoprotein were not completely solubilized from the membrane, other microsomal proteins would be immunoprecipitated with it making it difficult to assess specificity of the antibody. Because proteins differ in their ability to be solubilized from membranes by detergents, this is only a problem with some membrane proteins. For example, in the case of NADPH-cytochrome c reductase a concentration of detergent which would completely solubilize this protein from microsomes and still allow immunoprecipitation of this enzyme by its antibody was easily found (studies presented in chapter two). It has been previously observed by others (60), however, that cytochrome P_{450} requires higher detergent concentrations to solubilize it from microsomes than does NADPH-cytochrome c reductase and this became a problem in trying to specifically immunoprecipitate cytochrome P_{450} hemoproteins from detergent-solubilized microsomes. A simple solution was found, however, by using the partially purified cytochrome P_{450} preparations. Although the protein constituents present in these fractions are part of a 350,000 dalton aggregate (167), they were found to be much more easily solubilized from one another, perhaps because during the isolation of these fractions a great deal of the membrane lipid is removed (66). Therefore hemoprotein 3 could be solubilized free of other membrane constituents at detergent concentrations which still allowed antigen-antibody interaction. Furthermore, since these fractions contained most of the other major membrane constituents present in microsomes and, in the case of the fractions isolated from the liver microsomes of control and 3-MC-pretreated rats,

all the hemoproteins thought to be multiple forms of cytochrome P_{450} (Figure 27, chapter three), they provided an adequate control to assess the specificity of the antibody towards hemoprotein 3.

It was observed from immunoprecipitation studies carried out using the antibody to hemoprotein 3 and detergent-solubilized partially purified cytochrome P_{450} fractions isolated from the liver microsomes of PB-pretreated rats that the antibody specifically precipitated hemoprotein 3 from the other proteins present in this sample. As previously discussed, experiments which were conducted to further analyze the hemoprotein 3 antigen used for antibody production suggested that it was a protein aggregate which might also contain hydrophobic peptide fragments resulting from the proteolysis of other membrane proteins originally present in the cytochrome P_{450}-enriched fraction used in its preparation. From immunoprecipitation studies, however, it would appear that if such peptides were present, they did not interfere with the preparation of an antibody which was specific for hemoprotein 3. Also, since the cytochrome P_{450}-enriched fraction treated with trypsin to prepare the hemoprotein 3 antigen contained major components having molecular weights greater than 45,000 daltons (Figure 31), it was of concern that during proteolysis these proteins might be degraded to 45,000 dalton polypeptides which would migrate on SDS-gels in the same position as hemoprotein 3. Thus it could appear by SDS-gel electrophoresis that a pure preparation of hemoprotein 3 had been obtained by the proteolysis procedure when in actuality, the preparation also contained large fragments of other microsomal proteins. Again, the specificity of the antibody for hemoprotein 3 in immunoprecipitation experiments conducted against

detergent-solubilized cytochrome P_{450} fractions isolated from PB-pretreated rats argues against this possibility, however.

The antibody prepared to hemoprotein 3 not only will specifically immunoprecipitate this component from detergent-solubilized cytochrome P_{450} fractions isolated from the liver microsomes of PB-pretreated rats, but it also was shown to specifically immunoprecipitate this 45,000 dalton component from cytochrome P_{450} fractions prepared from the liver microsomes of control and 3-MC-pretreated rats. This suggests that the antibody does not cross-react with either hemoprotein 1 or 2 which are thought to be different forms of cytochrome P_{450} and which are major components of the partially purified cytochrome fractions isolated from control and 3-MC-pretreated rats. This would be a very important characteristic if the antibody were to be a potential tool for specifically studying the hydroxylation reactions catalyzed by hemoprotein 3. Since it is known that antibodies can cross-react with proteins having similar antigenic propteries and still not immunoprecipitate the proteins, however, it was thought that further studies on the specificity of this antibody were merited. For this reason Linda Chaney is developing complement fixation assay which can be used to study the specificity of the binding of the antibody to liver microsomes from control and PB- or 3-MC-pretreated rats. In theory, the amount of complement fixed by these microsomes should be directly related to the amount of antibody bound (172). Thus if the antibody is specific for the 45,000 dalton hemoprotein induced in rat liver microsomes by PB-pretreatment, more complement should be fixed to the liver microsomes isolated from rats pretreated with this compound than to the liver microsomes isolated from control or

3-MC-pretreated rats. Ms. Chaney has preliminary data to suggest that at a constant concentration of microsomal protein, the microsomes from PB-pretreated rats bind 2.8 times as much antibody as do the microsomes from control animals while the microsomes isolated from 3-MC-pretreated animals bind 1.9 times the amount those from control rats do. If Figures 22 and 23 (chapter three) are used to estimate, by integration of peak sizes, the amount of hemoprotein 3 (protein 6 in these figures) present in these three types of microsomes, it can be determined that the microsomes from PB-pretreated rats appear to contain 3 times as much of this protein as do the microsomes from control rats while the microsomes from 3-MC-pretreated rats contain approximately 2 times as much as do control. The correspondence of the amount of complement fixed by the three types of microsomes with the amount of hemoprotein 3 present in each type is striking and further argues for the specificity of the antibody.

Also, data obtained by Fredrick O'Neal in studying the ability of the antibody to inhibit microsomal hydroxylation reactions catalyzed by a solubilized reconstituted microsomal hydroxylation system similar to that described by Lu et al. (73,74) has suggested that the antibody will inhibit reactions preferentially catalyzed by partially purified cytochrome P_{450} fractions isolated from PB-pretreated rats much more effectively than it inhibits reactions catalyzed preferentially by partially purified preparations of cytochrome P_{448} isolated from the liver microsomes of 3-MC-pretreated rats. Thus the antibody will inhibit the N-demethylation of benzphetamine catalyzed by the cytochrome P_{450} fraction isolated from the liver microsomes of PB-pretreated rats while it will not inhibit benzpyrene hydroxylation

catalyzed by the cytochrome P_{448} fraction isolated from the liver microsomes of 3-MC-pretreated rats. Previously performed SDS-gel electrophoresis analyses of these cytochrome P_{450} preparations has indicated that the preparation isolated from the liver microsomes of PB-pretreated rats is greatly enriched in hemoprotein 3 while the 3-MC-preparation is enriched in hemoprotein 1 (Figure 27, chapter three). Therefore, such an inhibition pattern would be expected if the different hemoproteins induced by PB and 3-MC were responsible for the apparently different substrate specificites of the cytochrome P_{450} fractions and the antibody were specific for only hemoprotein 3. The results of these preliminary inhibition studies are quite exciting since they suggest that the antibody does specifically inhibit hydroxylation reactions catalyzed by hemoprotein 3 and therefore may be an important tool in studying the microsomal hydroxylation activities catalyzed by this hemoprotein.

As suggested previously in the discussion to chapter three, one would expect there to be a great deal of homology between the three hemoproteins, thought to be multiple forms of cytochrome P_{450}. For this reason, the apparent specificity of the antibody towards hemoprotein 3 is quite surprising since it indicates that the structures of these hemoproteins may be quite distinct. In exploratory experiments conducted in collaboration with Robert Moore in Dr. Aust's laboratory, it has been observed that hemoproteins 1, 2, and 3, which are all present in a cytochrome P_{448}-enriched fraction isolated from the liver microsomes of a 3-MC-pretreated rat, also have different susceptibilities to proteolysis by trypsin. Thus proteins 1 and 2 are readily digested by this protease while hemoprotein 3 is not. This observation,

too, suggests that the three hemoproteins have quite different structures. It could be argued that since hemoproteins 1 and 2 have higher molecular weights than hemoprotein 3 perhaps all three hemoproteins have only slightly different structures and trypsin converts the two higher molecular weight hemoproteins into the lower molecular weight form. If this were the case, however, one would not expect them to respond so differently in immunoprecipitation studies using antibody to hemoprotein 3. Clearly more experimentation on this subject is needed and hopefully, in the future, techniques will be found such that the three hemoproteins can be isolated from microsomes in their native forms. Then more definitive studies can be pursued in their structural differences.

The preliminary results of collaborative experiments which are being conducted with Linda Chaney and will be described more thoroughly in her Masters Degree Thesis have also yielded some information on the orientation of hemoprotein 3 in the rat liver microsomal membrane. In these experiments it has been found that portions of this hemoprotein are exposed to the exterior of the membrane since the antibody will bind to the membrane as assayed by complement fixation and membrane agglutination studies. This observation in fact confirms the suggestion made in chapter three that this hemoprotein, which is induced by PB-pretreatment, has an exterior location on the membrane. This previous suggestion had been made on the basis of a comparison of the amount of ^{125}I incorporated into 50,000 molecular weight proteins in the liver microsomes from control and PB-pretreated rats, after lactoperoxidase-catalyzed protein iodination. Ms. Chaney has also found that the antibody to hemoprotein 3 will not inhibit mixed-function

oxidase hydroxylation reactions catalyzed by the microsomes isolated from PB-pretreated rats, even under conditions where the microsomes are highly agglutinated. This suggests that, in the membrane, the active site of this hemoprotein cannot interact with the antibody either because it is buried within the tertiary structure of the protein or within the phospholipid bilayer of the membrane. The observations made by Fredrick O'Neal that the antibody can inhibit hydroxylation reactions catalyzed by a detergent-solubilized, reconstituted microsomal system, however, argue for the latter possibility. That is, the reactive site of this hemoprotein is probably in direct contact with the membrane lipids, and, indeed, such an orientation would seem logical because most of the substrates for the mixed-function oxidase system are lipophilic. Also this would be consistent with observations made by others (64) that the treatment of microsomes with phospholipases seems to alter the ability of substrates to bind to cytochrome P_{450}. In this light it should also be noted that even solubilized reconstituted microsomal hydroxylation systems have a phospholipid requirement (76). Perhaps this is because the phospholipid is required at the reactive site of cytochrome P_{450}.

Thus, the model which emerges from studies using the antibody to hemoprotein 3 to examine the orientation of this cytochrome P_{450} hemoprotein in the microsomal membrane is one in which the active site of this protein is located near or within the phospholipid bilayer of the membrane. This active site may then be surrounded on one side with portions of the cytochrome which are exposed to the exterior of the membrane and on the other side by portions buried within the phospholipid bilayer. This model suggests a different structure for

this protein than has been described for two other microsomal proteins which have been well characterized in regards to their position in this membrane. These two proteins are NADH-cytochrome b_5 reductase and cytochrome b_5 and their structures have been discussed in detail previously in this thesis. The active sites of these proteins are exposed to the exterior of the membrane and do not appear to interact with the membrane phospholipids (34,35). In fact the only portion of these proteins which appears to interact with the membrane phospholipids is a hydrophobic tail segment located at one end of the protein. It will therefore be interesting to see how the structure of hemoprotein 3 compares to those already described for NADH-cytochrome b_5 reductase and cytochrome b_5.

In this chapter then, the identification of the 45,000 dalton hemoprotein present in rat liver microsomes as a cytochrome P_{450} hemoprotein has been described. Antibody has been prepared to this hemoprotein and appears to be specific for this protein. Preliminary data are currently being accumulated by other members of Dr. Aust's laboratory which suggest that this antibody will be an important tool in studying microsomal hydroxylations catalyzed by this hemoprotein and also its orientation within the microsomal membrane. Hopefully techniques can also be found in the near future to isolate the 53,000 and 50,000 hemoproteins so that experiments can be performed to access their relationship to the mixed-function oxidase system and compare their structures to that of the 45,000 dalton hemoprotein.

SUMMARY

The experimental work detailed in this thesis began by utilizing the techniques of SDS-polyacrylamide gel electrophoresis and lactoperoxidase-catalyzed protein iodination to examine the general arrangement of proteins within the rat liver endoplasmic reticulum membrane. Lactoperoxidase-catalyzed protein iodination was carried out in the presence of the antioxidant, BHT, to prevent the peroxidation of membrane lipids during the procedure. It was found that the two subfractions of this organelle, the rough and smooth endoplasmic reticulum, have similar protein compositions after they are washed free of ribosomes and adsorbed cytoplasmic proteins using 0.3 M sucrose containing 0.1 M sodium pyrophosphate, pH 7.5. In each case the major polypeptide components have molecular weights ranging between 40,000 to 60,000 daltons while the minor components of the membrane span the range between 10,000 to over 200,000 daltons. Many minor polypeptide components of the membrane having both high and low molecular weights and major protein components of molecular weight 50,000 are located on its exterior (cytoplasmic face).

Further studies were then conducted using these techniques to more specifically examine the protein components associated with the microsomal mixed-function oxidase electron transport system. Based

upon the results obtained from such investigations, this electron transport system seems to be composed of a single form of NADPH-cytochrome c reductase enzyme which transfers electrons from NADPH to at least three different cytochrome P_{450} hemoproteins. The reductase enzyme appears to be a minor component of all the microsomal protein while the cytochrome P_{450} hemoproteins are major components. It therefore appears that during microsomal hydroxylations a single NADPH-cytochrome c reductase enzyme is responsible for supplying electrons to several cytochrome P_{450}'s.

NADPH-cytochrome c reductase is located in the exterior (or cytoplasmic face) of the microsomal membrane. This enzyme appears, by SDS-polyacrylamide gel electrophoresis, to have a molecular weight of 79,000 daltons. A major portion of this enzyme can be cleaved from the microsomal membrane by the action of proteases. This portion, which has a molecular weight of 71,000 daltons on SDS-polyacrylamide gels, contains the active site of the enzyme. On this basis it is hypothesized that NADPH-cytochrome c reductase may be an amphipathic protein. Thus the major portion of this enzyme, which contains the active site, is probably hydrophilic in nature and is attached to the microsomal membrane through a hydrophobic "tail" of approximately 70 amino acids.

The three types of cytochrome P_{450} hemoproteins can be distinguished by their migration properties on SDS-polyacrylamide gels. By this criterion, the three hemoproteins migrate with apparent molecular weights of 53,000, 50,000, and 45,000 daltons. The 53,000 dalton hemoprotein is induced in those microsomes isolated from rats pretreated with 3-MC while the 45,000 dalton cytochrome is induced by PB-pretreatment. These three forms of cytochrome P_{450} are hypothesized

to have different specificites for the substrates known to be hydroxylated by the mixed-function oxidase system. The different cytochrome P_{450} hemoproteins are therefore probably the mechanism by which the multiple mixed-function oxidase activities are attained. Like NADPH-cytochrome c, reductase, portions of the cytochrome P_{450} hemoproteins are probably also located on the exterior of the microsomal membrane. This is suggested by the observation that during lactoperoxidase-catalyzed protein iodination, the liver microsomes isolated from 3-MC- and PB-pretreated rats incorporate more ^{125}I into membrane proteins having molecular weights of approximately 50,000 daltons than do control microsomes. Since cytochrome P_{450} hemoproteins induced by these compounds are major microsomal proteins having molecular weights of approximately 50,000, one explanation for the increase in ^{125}I-incorporation would be that these proteins are located on the exterior of the membrane.

The 45,000 dalton hemoprotein is not detectably degraded by the proteolytic action of trypsin (assayed by SDS-polyacrylamide gel electrophoresis) and this characteristic was used to isolate it from other microsomal constituents. The hemoprotein thus isolated gave a cytochrome P_{420} spectrum suggesting that it was an altered cytochrome P_{450} hemoprotein. Antibody was prepared to this hemoprotein and this antibody appeared, on the basis of immunoprecipitation studies, to be specific for only the 45,000 dalton hemoprotein. This suggests that the three hemoproteins, thought to be multiple forms of cytochrome P_{450}, differ antigenically as well as in apparent molecular weights on SDS-gels. Preliminary studies indicate that the antibody to the 45,000 dalton may be an important mechanism through which to study

reactions catalyzed by this hemoprotein. It may also be an instrument through which to study the position of this hemoprotein within the microsomal membrane.

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APPENDIX

APPENDIX

List of Publications

Ann F. Welton and S. D. Aust, "Lipid Peroxidation During Enzymatic Iodination of Rat Liver Endoplasmic Reticulum," Biochem. Biophys. Res. Commun., 49, 661-666 (1972).

Ann F. Welton, Thomas C. Pederson, John A. Buege, and Steven D. Aust, "The Molecular Weight of NADPH-Cytochrome c Reductase Isolated by Immunoprecipitation from Detergent-solubilized Rat Liver Microsomes," Biochem. Biophys. Res. Commun., 54, 161-167 (1973).

Ann F. Welton and Steven D. Aust, "Multiplicity of Cytochrome P450 Hemoproteins in Rat Liver Microsomes," Biochem. Biophys. Res. Commun., 56, 898-906 (1974).

In Press

Ann F. Welton and Steven D. Aust, "The Effects of 3-Methylcholanthrene and Phenobarbital Induction on the Structure of the Rat Liver Endoplasmic Reticulum," Biochim. Biophys. Acta.

In Review

Ann F. Welton, Frederick O'Neal, and Steven D. Aust, "Multiplicity of Cytochrome P450 Hemoproteins in Rat Liver Microsomes: I. Purification and Preparation of Antibody to the Hemoprotein Induced by Phenobarbital," J. Biol. Chem.

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Abstracts

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