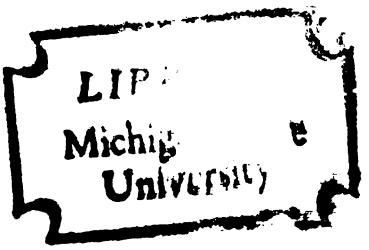
STUDIES ON THE RESOLUTION OF MICROSOMAL MEMBRANE PROTEINS

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
FREDRICK OLIVER O'NEAL
1969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ABSTRACT

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By

Fredrick Oliver O'Neal

The effects of treatments, such as organic solvent and salt extractions, lysis in the presence of 2.76% glycerol, and washing with buffer containing 0.01 M EDTA and 0.15 M KCl, on the resolution of microsomal membrane proteins were investigated. The proteins extracted by these treatments were identified by polyacrylamide disc gel electrophoresis and found to be involved in either hydrophobic or electrostatic (e.g. ribosomal proteins) interactions with the microsomal membrane. A relationship between the extent of protein solubilization and the polarity of the organic solvents used in the extractions was observed. Similarly, a relationship was observed between protein solubilization and the extent to which salts containing chaotropic ions (i.e., those which favor the transfer of apolar groups to water such as SCN^-, Br^-, NO_3^-, and Cl^-) changed the structure and lipophilicity of water. The harshness of these treatments (lysis, EDTA-KCl wash, salt extractions, and freezing in the presence of 50%

glycerol and 0.25 M sucrose) was evaluated by determining their effects on NADPH-cytochrome C reductase and aminopyrine demethylase activity.

Protein fractions were isolated from microsomes which may correspond in function to the apparently non-catalytic, structural proteins of mitochondria. One fraction is that isolated by detergent treatments and classically termed "Structural Protein"; the other, identified here by its being the predominate protein species in the electrophoresis profile of microsomal membranes, termed "Core protein." The former is believed to be functionally identical to the structure determining components of the headpieces and the latter that of the basepieces of mitochondrial membranes. These findings give support to more recently accepted membrane model in which membranes are thought to be composed of lipoprotein repeating subunits.

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Fredrick Oliver O'Neal

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Biochemistry

1969

ACKNOWLEDGMENTS

The author wishes to thank Dr. Steven D. Aust for his guidance and encouragement throughout the completion of this work. Also the author would like to thank Drs. Deal, Suelter, and Wilson and members of the Aust Lab for their helpful suggestions and/or use of equipment.

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LIST OF ABBREVIATIONS

ATPase	Adenosine Triphosphatase
CP	Core protein
DOC	Sodium deoxycholate
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
HBHM	Heavy beef heart mitochondria
HBLM	Heavy beef liver mitochondria
NADPH	Nicotine adenine dinucleotide phosphate, reduced
RER	Rough endoplasmic reticulum
RNA-ribose	Ribose determined from the hydrolysate of RNA
SER	Smooth endoplasmic reticulum
SER I	Mg^{++} binding subfraction of SER
SER II	Non-Mg binding subfractions of SER
SP	Structural protein
Tris-HCl buffer	0.05 M Tris-hydroxymethylaminomethane, pH 7.5

INTRODUCTION

The study of biological membranes has been an intense field of research for a number of years. Attempts to define these membranes with respect to function and morphology have led scientists through the years to propose models, each of which was representative of the data available. One such model which has been widely accepted was that proposed by Danielli and Davson (1) and later extended by Robertson (2), which emphasized the function of biological membranes as being passive barriers to free diffusion and electrical insulators. This model was pictured as having an interior bimolecular leaflet of phospholipid, held together by Van der Waals attractions between apolar regions of phospholipid, with proteins relegated to the exterior interacting with the polar groups of phospholipid. Evidence, however, has been accumulating within the past few years which disproves the assumptions upon which the classical model was based, suggesting a model in which membranes are represented as expressions of macromolecular lipoprotein subunits (3, 4). These evidences have been reviewed (5, 6, 7, 8) and will not be enumerated here; however, mention should be made of the pioneering work of Fernandez-Moran (9) whose negative

staining techniques for electron microscopy provided concrete evidence for the subunit structure in membranes.

It is interesting that Green et al. (10) were able to isolate a protein fraction from mitochondria by the use of bile salts and the detergent sodium dodecyl sulfate with properties (10, 11, 12) which makes it plausible to designate it as being the repeating unit in membranes. Specifically, this protein possessed the following properties: insolubility at physiological pH; the ability to form stable complexes with itself, with enzymes indigenous to the mitochondria, e.g. cytochromes a, b, and c, and with lipid; an apparent noncatalytic function; and it is the predominate protein species in the mitochondrion. In like manner proteins with similar properties have been isolated from other membrane systems (13, 14, 15), indicating the existence of a universal protein or class of proteins which function as the structural proteins of membranes.

Attempts to study the properties of structural proteins have led to the discovery that the isolation procedures of Green et al. (10) or its more popular modification by Richardson (13) yield a grossly heterogenous protein fraction, as was determined by the electrophoresis system of Takayama (16). Recently, however, purifications of this crude structural protein has been achieved by Lenaz et al. (17) with acidic methanol extractions followed by extractions with 8 M urea (pH 5.5). Though effective, these

methods were shown to yield a damaged protein species (18). It is unfortunate that the technology for resolving membrane proteins has not advanced to the level where such proteins can be rendered pure and in a state analogous to that *in vivo*. Accurate characterization of the physiological and functional properties of membrane proteins, whether their function is enzymatic or structural, demands the use of milder techniques for membrane resolution. It was with this premise in mind that the present study on the resolution of liver endoplasmic reticulum (ER) membranes into structural and other protein fractions was undertaken.

Attempts have been made during this study to resolve liver ER membranes by the use of organic solvent extractions and salt extractions. Of these methods, salt extractions of ER vesicles, following lysis to remove their soluble contents, were more promising because of their relative mildness and in some cases specificity with respect to protein solubilization. The structural protein content of the various membrane protein fractions was determined by its electrophoretic mobility on the polyacrylamide gel electrophoresis system of Takayama (16). Identification of structural protein as being the most abundant membrane protein species in the electrophoresis profile of whole membranes can be rationalized because of the fact that it was found, as isolated by Green *et al*. (10), to represent approximately

55% of the particulate protein of mitochondrial membranes. It must be mentioned, however, that designation of that protein fraction, possessing such properties, as structural protein may be erroneous in that a structural function has yet to be demonstrated.

In working with endoplasmic reticulum membranes one has to be concerned about contamination by ribosomes which in themselves contain a rather heterogenous class of proteins. Since difficulties were encountered in obtaining sufficient quantities of smooth or ribosome free endoplasmic reticulum membranes, precautions were taken to remove ribosomes. Chief among these methods were lysis of vesicles and an EDTA wash, which judging by the RNA content of the extracted membranes were somewhat effective. Attempts to chemically determine the extent of removal of non-structural protein from the membrane proteins was done by monitoring lipid phosphorus content of extracted membrane preparation. The rationale behind this procedure, which is in accord with the membrane model posed earlier, is that membranes are expressions of macromolecular lipoprotein repeating units (3, 4).

LITERATURE REVIEW

Membranes

Resolution of the proteins of membranes to study their physical, chemical, and functional properties presupposes that one has at least a working model. The classical model of membranes is the paucimolecular model of Danielli and Davson (1) picturing membranes as consisting of one or more bimolecular phospholipid leaflets sandwiched between two layers of protein in the extended or beta conformation. Both the lipid and protein form continuous and separate phases, the latter being electrostatically bonded to the polar groups of phospholipids. Initially the model proposed that there were two bimolecular phospholipid layers separated by neutral lipids, however, this view in light of electron microscopic and X-ray diffraction studies (19, 20) of membranes, was revised to that of the unit membrane hypothesis of Robertson (2). The major difference between the two being that the observed thickness of membranes could accommodate only one bimolecular phospholipid layer between the two protein layers. Though this model was supported also by the chemical composition of some membranes, e.g. lipid content of membranes range from 30% for mitochondria to 80% by

weight for myelin (14), further experiments using improved techniques bore data contrary to the classical model. The suggested role of lipid was first shown to be a limitation of the model when it was discovered that the binding mode of lipid to protein was hydrophobic rather than electrostatic (21, 22, 23). More alarming were the studies of Fleischer et al. (24) who were able to extract up to 85% of the lipid from mitochondrial membranes observing essentially no alterations in the trilaminar structure characteristic of electronmicrographs of membranes fixed with osomium tetroxide. The fact that the membrane did not collapse thus shifted the role of the structural determinant of membranes from lipids to proteins. Another argument against the classical membrane model is that about 20 to 50% of total membrane proteins, which were observed by Infrared Spectroscopy, Circular Dichroism, and Optical Rotatory Dispersion (22, 25, 26) were shown to be present partly in the alpha helical conformation and the rest predominantly in a random coil conformation rather than the extended or beta conformation as predicted by the model. Thus the classical membrane model seems to be based upon observations which have been shown to be circumstantial and not in accord with chemical and physical observations.

Over the last decade evidence has been accumulating indicating that the structural components of membranes are macromolecular lipoprotein subunits and that membranes are formed by repeating subunit layers one unit thick. The

first indications of the subunit structure of membranes were suggested by the observations of Green (27) and others (28, 29, 30) that the enzymatic systems of electron transfer and the citric acid cycle in mitochondria appeared to be an ordered arrangement of macromolecular enzyme complexes. Similar observations have also been made for endoplasmic reticulum (31) and chloroplast membranes. That the ultrastructure of membranes consisted of macromolecular repeating units was first shown in electron micrographs of mitochondria (9) and later in microsomes (31), bacterial membranes (32, 33), and plasma membranes of liver cells (34) using the negative staining techniques of Fernandez-Moran (9). Other techniques of electron microscopy, e.g. the freeze etching technique of Moor and Muchlethaler (35), have also demonstrated the repeating subunit structure of membranes thus eliminating the possibility of artifacts due to sample preparation.

Studies on the inner mitochondrial membrane have provided evidence correlating the macromolecular subunits of this membrane with the enzymatic functions of electron transport. This membrane system has been shown to consist of tripartite repeating units (9), i.e., an invarient membrane forming sector or basepiece, a variant or detachable section consisting of a headpiece and stalk. The enzymatic functions of electron transport have been resolved into four electron transport complexes: I, NADPH-Coenzyme Q

reductase (28); II, Succinate-Coenzyme Q reductase (3); III, Reduced Coenzyme Q-cytochrome c reductase (36); and IV, cytochrome C oxidase (37). Each of these functions has been associated with the basepieces of the inner mitochondrial membrane (38). Most interesting have been the membrane reconstitution studies by Green (38) and Kopaczyk (39) using these electron transport complexes. They were able to show that the inner mitochondrial membrane or any of the complexes taken singly or in different combinations could, after being depolymerized with bile salts, form membrane vesicles upon removal of the bile salts. In like manner complexes II and III, when prepared by methods which remove structural protein (see the introduction or section II of this review for definition), possessed the same membrane forming capabilities (39), suggesting a structural role for catalytic proteins. One function of lipid which resulted from these reconstitution studies was that of regulation of the mode of membrane formation. Lipid, distributed on two faces of the cuboidal shaped basepieces, inhibits binding of other basepieces at these faces and assures that membranes are formed as two dimensional continuums one subunit thick (3). Lastly, formation of membranes in these reconstitution experiments was shown to be specific for the four electron transport complexes, other molecules tested including structural protein and the headpieces of the inner mitochondrial

membrane did not show this membrane forming ability. This latter finding warrants a reconsideration of the function of structural protein in membrane systems.

Thus the repeating subunit structure is, in light of the evidence stated previously, an attractive working model. In most membranes which have been observed by negative staining techniques of electronmicroscopy, such a model seems applicable; what remains to be done is to define the repeating units of biological membranes with respect to organization, enzymatic activity, and the function of that most abundant protein fraction within these subunits, structural protein.

Structural Protein

Structural protein (SP) is that water insoluble and apparently noncatalytic protein which was designated by Green et al. (10) as being the protein constituent of the lipoprotein subunits of mitochondrial membranes. Its isolation was first achieved by solubilization of membranes with bile salts (deoxycholate and cholate at concentrations of 2 mg/mg protein and 1 mg/mg protein respectively) and sodium dodecyl sulfate (0.75 mg/mg protein), collecting the protein which precipitates from 0-12% ammonium sulfate concentration, and extracting lipid and bile salts with either 75% methanol at 50°C or as modified by Richardson (13), with 90% acetone. The resulting protein fraction, which shall be designated here as "crude SP," was

characterized physically and chemically (10, 12, 40) and prematurely assigned the role of being solely responsible for membrane integrity. Crude mitochondrial SP was shown to have the following properties which gives support to its designated function as mentioned above: it was found to be the major protein constituent of the mitochondria and it has the ability to bind and form stable complexes with lipid and enzymes of the mitochondrial electron transfer chain. Other properties which further suggest a structural rather than catalytic role include the fact that it contains no enzyme cofactors such as flavin, heme, nonheme iron, or copper and was found to be soluble only in detergent solutions at extremes of pH.

Criddel et al. (40, 41) initially were able to obtain both physical and chemical evidence suggesting that crude SP as isolated by the above methods (10, 13) was homogeneous. Sedimentation studies of this crude SP fraction solubilized in a solution containing 0.1% sodium dodecyl sulfate and 0.1 M NaCl at pH 10.5 showed a single symmetrically migrating boundary with sedimentation coefficient corresponding a molecular weight of roughly 22,000. Further studies revealed: the presence of only one carboxy terminal amino acid; that one protein band is observed upon starch gel electrophoresis of SP dissolved in 0.3% sodium dodecyl sulfate at alkaline pH; and peptide mapping of trypsin digests of crude SP gave close correlations between the number of peptides observed and the

number of lysine plus arginine residues present. However, subsequent work by Lenaz et al. (17), who were able to purify SP from a crude SP preparation isolated by the conventional method by extractions with 0.4% TCA in methanol and 8 M urea, did not confirm the homogeneity of that protein fraction termed here as crude SP. To date the heterogeneity of this crude SP fraction has been shown by several criteria: electrophoresis of crude SP by the method of Takayama (16), in which samples are dissolved in a phenol-acetic acid solution and run on polyacrylamide gels which contain 5 M urea and 35% acetic acid, showed multiple bands; several N-terminal amino acid species were detected when dinitrophenylation of crude SP was performed in 6 M guanidine hydrochloride (18); and multiple peaks resulted from sedimentation velocity studies of crude SP in the presence of 6 M guanidine hydrochloride (42). These findings question the homogeneity of the crude SP fraction of Green et al. (10) and gives cause for critical review of some of the assumptions made regarding its properties and function.

Purification of SP from heavy beef heart mitochondria (HBHM) has recently been achieved by Lenaz et al. (17). Their method essentially involves extraction of crude SP, prepared according to Green (10), Richardson (13) or modifications thereof (17), with 0.4% TCA in methanol followed by extractions with 8 M urea at pH 5.5. Though

this method has been shown to damage the proteins (18), it is thus far the only method shown effective in purifying SP. One interesting aspect of these studies has been the correlation of the solubility properties of the crude SP with the method of isolation which indicates differences in conformational states of these crude SP preparations. Some physical properties of purified SP isolated from HBHM, heavy beef liver mitochondria (HBLM), HBHM ATPase, and HBHM outer membrane have been determined and found to be quite similar (18, 43). These properties include: the fact that the average molecular weights all fall within the range of 60,000 to 70,000; the peptide maps of tryptic digests of these SP species are similar, suggesting structural similarities; amino acid compositions of these SP species were strikingly similar whereas in comparison to those for the other enzymes associated with these membranes were markedly different; and the N-terminal amino acids of these pure SP preparations were found to be either aspartate or alanine. Differences were found to exist in the number of SP species seen on polyacrylamide gel electrophoresis after oxidation of pure SP isolated from different membranes (17, 43). Some correlations have been made as to the specific locations of these species within the mitochondrion, e.g. two species have been located in the outer and two in the inner mitochondrial membrane, and as to the small but consistent differences in the amino acid contents of the species located in the outer as compared to

those located in the inner mitochondrial membrane (18). These properties argue against a single structural protein species within a given membrane system and suggests that structural protein forms a class of noncatalytic proteins.

There now appears to be other species of noncatalytic proteins associated with mitochondria which have been recently designated by Green et al. (43) as being core proteins (CP). Evidence has been accumulating which suggests different locations for the two types of noncatalytic proteins within the tripartite repeating units (9) of the mitochondrial membrane, i.e., SP has been shown to be the noncatalytic protein of the detachable sectors whereas CP is that associated with the basepieces. An example of such evidence is the recent finding that a protein fraction, which is electrophoretically identical to Green's SP (10, 43), could be extracted with 1.4% acetic acid or 7 M urea from mitochondria (44) and submitochondrial vesicles (45) without destroying membrane integrity as evidenced by electronmicroscopy. These findings coupled with the fact that core proteins have been shown to account for some 50 per cent (35, 39) of the protein in basepieces (complexes III and IV) of the inner mitochondrial membrane adds further proof to Green's premise (43) regarding the location of noncatalytic proteins within the mitochondrion. Each of these noncatalytic proteins were shown to be physically similar, the only exceptions being that core proteins are

resistant to hydrolysis by proteolytic enzymes, e.g. trypsin, pronase, and papain (43), and that the molecular weights of CP fell within the range of 50,000 to 51,000 as opposed to 60,000 to 70,000 found for SP (18, 43). In light of these findings, there appears to exist within the mitochondrial membranes a general class of non-catalytic proteins of which SP and CP form subclasses. Their locations within the mitochondrial membrane have thus been established but their location in other membranes and their specific functions have yet to be demonstrated.

METHODS AND MATERIALS

Chemicals

Most chemicals used in these experiments were of reagent grade from the usual sources and underwent no further purification, unless mentioned otherwise. Sodium dodecyl sulfate, ribonuclease-A (proteinase free), the sodium salts of cholic and deoxycholic acids, NADPH, $NADP^+$, isocitric dehydrogenase, D,L-isocitric acid, and cytochrome c (horse heart) were all obtained from Sigma Chemical Co., St. Louis, Mo. The chemicals used in polyacrylamide gel preparation (acrylamide, N,N^1-methylene bisacrylamide, N,N,N^1,N^1-tetramethylethylenediamine and ammonium persulfate) were obtained from Canalco Industrial Co., Rockville, Md. Aminopyrine was obtained from K and K Laboratories, Inc., Plainview, N. Y. From Eastman Organic Chemical, Distillation Products Ind., Rochester 3, N. Y., 2,4-pentanedione was obtained. Orcinol was obtained from HARLECO, Philadelphia, Pa., and purified by dissolving in boiling benzene, decolarizing with charcoal, and recrystallized by cooling the solution.

Animals

The rats used in these studies, weighing from 200 to 350 grams, were of the Holtzman strain obtained from

Spartan Research, Haslett, Michigan. Unless mentioned otherwise, rats of either sex were used indiscriminately. In some experiments where proliferation of smooth endoplasmic reticulum of the liver was desired, rats were treated with phenobarbital. The dosage of phenobarbital (100 mg/kg) was given daily by intraperitoneil injections five days prior to sacrifice, or was contained in the rats' drinking water at 0.1% concentration for a period of at least 14 days prior to sacrifice. Beef livers were obtained fresh from Van Alstein Packing Co., East Lansing, Mich.

Isolation of Microsomes

Method 1.--Fresh beef or rat livers were minced, added to three volumes of cold 1.15% KCl, and homogenized with ten strokes in a Potter-Elvehjem homogenizer, clearance of 0.0069 inches, fitted with a motor driven teflon pestle. The resulting homogenate was centrifuged for 20 minutes at 10,000g. The supernatant was carefully decanted and centrifuged for 100 minutes at 105,000g in the number 30 rotor of a Spinco Model L preparative ultracentrifuge. The 105,000g supernatant was discarded and walls of the tubes wiped free of lipid. A small amount of 1.15% KCl was added to each tube followed by gentle shaking of the tubes to separate the microsomal pellet from glycogen. The pellets were washed by resuspending in KCl (three volumes of initial liver weight) and centrifuging as

before. Pellets were separated from glycogen as described above and resuspended in a small volume of 0.05 M Tris-HCl buffer, containing 50% glycerol and stored at -15° until used.

Method 2.--Same as method 1 with the following exceptions: livers were homogenized in and the microsomes stored in 0.25 M sucrose; and separation of the microsomal pellet from glycogen could not be obtained.

All of the above procedures were performed at 0-4°. Rat livers were perfused, in situ, with the homogenizing solution until the livers were blanched. The livers were then excised from the rat and immediately placed on ice. Beef livers were packed in ice as soon as possible after sacrifice of the animal.

Lipid and RNA Extraction

Lipid and RNA were extracted from tissue homogenates essentially according to the method of Schneider (46). Acid soluble materials were removed by extraction of the protein sample with cold 10% TCA for 10 minutes. Lipid was then removed from the remaining pellet by extractions at room temperature for 20 minutes with 5 ml of 95% ethanol followed by another extraction with 3 ml of an ethanol-petroleum ether mixture (1:2). Extraction of RNA from the lipid free pellet was accomplished by hydrolysis of RNA with 1N NaOH for at least 20 hours at 37° followed by precipitation of protein and DNA by neutralization of the solution.

Isolation of Structural Protein

Structural protein was isolated from beef and rat liver microsomes essentially by the method of Criddle et al. (40) using modification "d" of Lenaz et al. (17). The method involves solubilization of microsomes with deoxycholate (2 mg/mg protein), cholate (1 mg/mg protein), and sodium dodecyl sulate (0.75 mg/mg protein) and reduction of cytochrome by addition of solid sodium dithionite followed by precipitation of structural protein by bringing the solution to 12% saturation with respect to ammonium sulfate. The solution is then allowed to stand for 16 hrs at 0-4° after which it is centrifuged for 20 minutes at 40,000g. The removal of lipid and bile salts from the pellet was accomplished by butanol extraction at 0-4° followed by a 75% methanol extraction at 50°. The solution was kept at pH 9.0 with 1.0 N NaOH during the addition of dithionite and ammonium sulfate.

Microsomal Fractionation

Fractionation of microsomes with 0.26% sodium deoxycholate (DOC).

--The procedure used here is essentially that of Ernster et al. (47) which involves the addition of 2.6% DOC (pH 8.0-8.5) stock solution to microsomes, suspended in Tris-HCl buffer to a protein concentration of approximately 6 mg/ml, contained in a 40 ml centrifuge tube. The tube's contents were gently mixed by inverting the tube several times and centrifuged at 105,000g for

40 minutes in a number 30 Spinco rotor. The smooth endoplasmic reticulum (SER) is contained in the loose, reddish, protein pellet whereas the rough endoplasmic reticulum (RER) plus some detached ribosomes are contained within the tight, light brownish, protein pellet formed as a result of the centrifugation. These fractions were separated, lyophilized, and assayed for their lipid-phosphate and RNA-ribose content.

Fractionation of microsomes on a discontinuous sucrose density gradient in the presence of CsCl and $MgCl_2$.--The method described here, which fractionates microsomal membrane vesicles according to their ability to bind Cs^+ and Mg^{++} ions, is that of Dallner et al. (48). To the 10,000g supernatant of rat livers homogenized in three volumes of 0.25 M sucrose (see microsome isolation Method 2 in Methods and Materials) enough of a 1 M CsCl stock solution is added to give a final concentration of 0.015 M, 4.5 ml of this is carefully layered over 2 ml of a 1.3 M sucrose, 0.015 M in CsCl contained within a 7 ml centrifuge tube. The tubes were then centrifuged for 2 hours at 105,000g in a number 40.2 Spinco rotor. Three fractions resulted from this centrifugation: a clear reddish supernatant; a cloudy white infranatant, located at the 0.25 M-1.3 M sucrose interface, which contains the bulk of the SER; and a tight, Cs^+ binding pellet containing RER. The SER and RER fractions were washed by suspension

in 1.15% KCl and centrifuged at 105,000g for 2 hrs, these fractions were designated as whole SER and RER, respectively.

Fractionation of SER was performed by diluting the infranatant or whole SER fraction from two of the tubes mentioned above with distilled water to a volume of 4.5 ml and adding 3 mg $MgCl_2$, final concentration 0.007 M. This is then layered over 2.0 ml of a 1.15 M sucrose, 0.007 M in $MgCl_2$ contained in a 7 ml centrifuge tube and is centrifuged in a number 40.2 Spinco rotor for 45 minutes at 105,000g. Three fractions resulted from this centrifugation: a clear supernatant; a magnesium binding tight pellet designated as SER I; and a magnesium non-binding infranatant protein fraction, designated as SER II. Both fractions were washed by suspension in Tris-HCl buffer and centrifuging for 90 minutes at 105,000g. The washed pellets in all cases were resuspended in Tris-HCl buffer and stored at 0-4° until ready for use.

Isolation of ribosomes.--Treatment of microsomes with 0.5% DOC according to the method of Palade and Siekevitz (49) was found to solubilize most of the membranes and constituent proteins with the exception of ribosomes. The procedure used was to add enough to a stock solution of 5% DOC to a microsomal suspension contained in a 40 ml centrifuge tube at a protein concentration of 10-20 mg/ml, to final DOC concentration of 0.5%; the contents of the tube

are then mixed by inversion several times and centrifuged for 100 minutes at 105,000g. The fractions obtained by this centrifugation were a clear reddish supernatant and a small pellet which contained ribosomes and possibly membrane fragments as contaminants. The latter was washed three times by resuspension in Tris-HCl buffer and centrifuging as mentioned above.

Fractionation of microsomes with tertiary-amyl alcohol--Isolation of electron transport membranes.--Fractionation of microsomes with tertiary-amyl alcohol was performed according to MacLennan et al (31). Microsomes suspended in 0.25 M sucrose to a protein concentration of 27 mg/ml were diluted with 1/3 volume of 0.9% KCl and tertiary-amyl alcohol was added to a final concentration of 10% by volume. The mixture is stirred slowly at room temperature for 10 minutes and is then centrifuged at 79,000g for 30 minutes at 4°. Three fractions should result; a clear supernatant; a loose reddish pellet containing the electron transport membranes; and a tight brownish pellet. Fractions were washed once by suspension in Tris-HCl buffer and centrifuging at 105,000g.

Microsomal Extraction Procedures

1.4% Acetic acid extraction.--The method of Zahler et al. (44) was used without modification. It involved: extraction of microsomal protein by suspending microsomes in 1.4% acetic acid (pH 3.1) to a protein concentration

of approximately 10 mg/ml; incubating the mixture in an ice bath for 30 minutes; and centrifuging at 105,000g for one hour. A protein fraction is then precipitated from the supernatant by adjusting the pH to 6.5 and collected by centrifuging for 30 minutes at 40,000g.

Lysis of microsomal vesicles.--

Method 1.--Microsomes are lysed at ice bath temperatures by thawing microsomes, if stored frozen, and diluting them to a final protein concentration of 1 to 3 mg/ml with cold distilled water. The mixture is then stirred in an ice bath for one hour after which the lysed vesicles are pelleted by centrifugation at 105,000g for 90 minutes.

Method 2.--This procedure is similar to that described in Method 1. The modifications were that lysis was performed at room temperature for 30 minutes and that glycerol was added to microsomes stored in 0.25 M sucrose to give a final concentration of 2.76% (approximately that concentration of glycerol obtained when microsomes stored in Tris-HCl buffer, 50% in glycerol are diluted 1 to 25 with distilled water to achieve lysis). In one experiment light scattering, as measured at 600 mμ on a Coleman spectrophotometer equipped with recorder, during lysis by this method was followed with time to get an idea of the time needed for complete lysis. Care was taken to ensure that any changes in light scattering were not due to protein settling out of the suspension.

Salt extractions of microsomes.--This method of extracting microsomal proteins involves addition of enough crystals of the appropriate salts to give the desired final salt concentration to 5 ml of Tris-HCl buffer at 0-4° contained in a 7 ml centrifuge tube. One milliliter of microsomal protein suspension (3 to 8 mg of the resuspended lysed pellet) is then added to each tube; the tubes are then capped and their contents mixed by inversion several times. The tubes were then incubated in an ice bath for one hour, with occasional mixing, after which they were centrifuged for two hours at 105,000g in a Spinco number 40.2 rotor. In each experiment a control, consisting only of microsomal protein in Tris-HCl buffer, was included.

Organic solvent extractions.--Microsomes which had been lysed and washed with pyrophosphate buffer (0.1 M followed by 0.02 M, pH 7.8) were lyophilized and either 5 or 100 mg samples extracted with organic solvents or solvent mixtures. Extraction of the lyophilized protein was performed by homogenizing in the solvent for three minutes with a teflon pestle fitted glass tissue homogenizer, followed by incubation in an ice bath for one hour with occasional mixing. The insoluble proteins were collected by centrifugation at 40,000g for 30 minutes and their protein content determined by the method of Lowry (50).

Sonic oscillation of microsomes.--A microsomal suspension of about 15-20 mg protein/ml in Tris-HCl buffer contained in a plastic tube was sonicated with a Bronson Sonic Power sonifier, with power scale turned to 4 D.C. amps. Sonication was carried out in an ice bath for intervals not exceeding 30 seconds, allowing one minute between intervals for cooling of the microsomal suspension. The sonicated suspension is then transferred quantitatively to a 7 ml centrifuge tube and the insoluble proteins removed by centrifugation at 105,000g for one hour.

Enzyme Assays

Aminopyrine demethylase.--Aminopyrine N-demethylase activity was assayed by measuring the rate of formaldehyde production according to the method of Nash (51). Fixed time assays of 7 minutes were used through these studies, unless mentioned otherwise. The incubation mixture used contained: $MgCl_2$ (7 mM), D,L-isocitrate (2mM), $NADP^+$ (0.1 mM), NADP-isocitrate dehydrogenase (0.05 units/ml), microsomes (0.5 to 1.2 mg protein/ml) and varying concentrations of aminopyrine (from $4.0X10^{-3}$M to $1.33X10^{-4}$M). The reaction is started by the addition of microsomes to the incubation mixture contained in a 20 milliliter beaker on a Dubnoff Metabolic Shaking Incubator at 37°. After a 7-minute period of incubation the reaction is stopped by addition of one milliliter aliquots of the incubation to one milliliter of 10% TCA contained in a 5 milliliter

centrifuge tube. Protein is allowed to precipitate for about 5 minutes and two milliliters of Nash reagent (2M $NH_4C_2H_3O_2$; 0.05 M CH_3COOH; and 0.002 M 2,4-pentanedione) are added. The mixture is then heated for 15 minutes at 50° to allow color development, centrifuged for about 5 minutes at 1000g, and the color of the supernatants read at 412 millimicrons in a Coleman Jr. Spectrophotometer equipped with a flow-cell of 1 cm. path length. An extinction coefficient of 7.08 OD. ml^{-1} of assay $\mu mole^{-1}$ of HCOH is used to calculate the µmoles of formaldehyde formation.

NADPH-Cytochrome c reductase.--NADPH-cytochrome c reductase activity was determined by the method of Omura et al. (52), which measures the initial rate of reduction of horse cytochrome c at 25°. The assay system contained 0.1 M phosphate buffer, pH 7.5, $1.3X10^{-5}M$ cytochrome c, $3.0X10^{-5}M$ NADPH, and microsomes (from 0.2 to 0.6 mg of protein) in a volume of 2.5 milliliters.

A millimolar extinction coefficient of $27.7X10^{-3}$ for reduced cytochrome c at 550 millimicrons was used to estimate reduced cytochrome c.

Analytical Methods

Protein was determined according to the method of Lowry et al. (50) at 750 mµ with crystallized bovine serum albumin as protein standard. Inorganic phosphate was determined according to the method of Bartlett (53), at

825 mμ. Total phosphate was determined by digestion of whole protein samples whereas lipid phosphorus was determined from the digests of the lipid extracts of protein samples. Ribose in the RNA extracts was determined by the orcinol method of Schneider (46).

Polyacrylamide Gel Electrophoresis

The disc electrophoresis system used was patterned after the method of Takayama (16). The final gels, 7.5% acrylamide in 35% acetic acid and 5 M in urea, were prepared by mixing stock solutions A and B with tetramethylethylenediamine in the proportions, 3:1:0.02(v/v). Stock solution A consisted of 6 grams acrylamide, 0.16 grams N,N^1-methylene bisacrylamide, 12 grams urea, 28 ml glacial acetic acid and water to make 60 ml final volume. Stock solution B consists of 12 grams urea and 0.3 grams ammonium persulfate in 20 ml water. Stock solution A could be stored for periods up to 6 months if kept refrigerated in a brown bottle; however, stock solution B was made fresh before each experiment. The buffer system used throughout was 10% acetic acid, both the cathode and anode. Polymerization of the gels was carried out in a water bath at 47° for 15 minutes. The gels were then covered with a solution containing 5 M urea in 75% acetic acid and pre-electrophoresed for one hour at 5 milliamps per tube to remove ammonium persulfate. The removal of persulfate from the gels by pre-electrophoresis was checked by

electrophoresing gels for various periods of time after which the gels were immersed in a solution containing 2% benzidine chloride in 10% acetic acid. The presence of persulfate was indicated by deep blue color formation, as observed by Bennick (54). All protein samples were dissolved (1 mg/ml) in a mixture containing phenol, acetic acid, and water (2:1:1) and from 0.1 to 0.15 mg applied to the gels, unless mentioned otherwise. Only the running gel of 7.5% acrylamide, 5.5 cm in length, was used. Electrophoresis was routinely performed at room temperature for one hour with a constant current of 5 milliamps per tube.

The gels were stained for a minimum of one hour in either Coomassie blue (0.05% in 12.5% TCA) or Amido Schwartz (0.55% in 7.5% acetic acid) and destaining by diffusion in 10% TCA and 7.5% acetic acid for gels stained with Comassie blue and Amido Schwartz, respectively. Ribonuclease-A was used as an internal standard.

Structural Protein Assay

Throughout these studies structural protein (SP) was identified as being the major protein species, i.e., the more densely stained protein band, in the polyacrylamide gel electrophoresis profile of untreated microsomes. The reasons for this designation are mentioned in the Introduction and Literature Review. The above definition of structural protein will be used throughout unless otherwise mentioned.

RESULTS

Fractionation of Microsomes

Fractionation with 0.26% DOC.--The problem of resolving liver microsomal membranes into their constituent proteins was first approached by investigating several methods known to separate the membranous from the non-membranous fractions of microsomes. A membrane preparation free of readily detachable proteins and ribosomes, which constitute a rather heterogenous protein class, was desired. Several methods were investigated, one of which was the treatment of microsomes with 0.26% DOC which, according to Ernster et al. (47), has previously been shown to yield a membrane fraction relatively free of attached ribosomes. Treatment of beef liver microsomes, isolated according to Method 1, by this method resulted in three protein fractions upon centrifugation: a clear yellowish supernatant; a loose, reddish, membranous pellet which should be essentially free of ribosomes; and a tight, brownish, protein pellet. It was found, however, by chemical analysis of the various fractions that the RNA-ribose content of the loose, reddish, membrane pellet had a higher RNA-ribose content than did the other fractions or untreated microsomes. This suggests a membrane contaminated by ribosomes.

Fractionation on discontinuous sucrose density gradient in the presence of Cs^+ and Mg^{++} ions.--Another fractionation method investigated was that which separates microsomes into RER, SER I, and SER II, on a discontinuous sucrose density gradient according to their ability to bind the cations, Mg^{++} and Cs^+. The chemical analysis of these three fractions and those of whole microsomes and whole SER are shown in Table 1 below.

TABLE 1.--Analyses of smooth and rough endoplasmic reticulum (SER and RER) from the livers of phenobarbital treated rats. [Separation of SER and RER and subfractionation of SER was achieved by centrifugation on a discontinuous sucrose gradient in the presence of CsCl and $MgCl_2$ (see Methods and Materials)].

Sample	Total-phosphate ($\frac{\mu mole}{mg\ protein}$)	Lipid-phosphate ($\frac{\mu mole}{mg\ protein}$)	RNA-ribose ($\frac{mg \times 10^{-3}}{mg\ protein}$)
Whole microsomes	3.31	2.56	15.75
RER	6.75	2.59	33.30
Whole SER	4.01	3.51	7.72
SER I	4.42	3.15	12.72
SER II	2.60	--	--

The fact that there is approximately a four-fold difference in the RNA ribose content of RER and whole SER indicates some degree of separation of these two fractions. Also the differences in the lipid-phosphate content of

whole microsomes and RER as compared to that of whole SER and SER I indicate that a degree of purification of membranes has taken place (keeping in mind the membrane model presented in the Introduction and Literature Review sections in which membranes are thought to be expressions of repeating lipoprotein subunits). In spite of the chemical differences between these membrane fractions, close similarities in the polyacrylamide gel electrophoresis patterns were observed. However, there were some differences in the electrophoresis profiles of the RER and SER II membrane fractions as shown in Figure 1 (tubes 2 and 5). The electrophoresis profile of RER, tube 2, shows the presence of faster migrating protein bands not seen in the other membrane fractions. It is likely that these faster migrating bands represent ribosomal proteins since it is generally assumed that the only difference between RER and SER is the presence of ribosomes on RER membranes.

The electrophoresis profile of SER II (Figure 1, tube 5) shows a prominent protein band which, though present in all other membrane fractions, seems to be more concentrated in this fraction. It was also observed that this membrane fraction had more of a reddish color than the other fractions, suggesting the identity of this band to be one of the microsomal cytochromes. Though the true identity of this protein band was not pursued, it is interesting to note the observation that this protein band is extractable

Fig. 1.--Fractionation of microsomes from phenobarbital treated rats with Mg^{++} and Cs^{+} ions.

1. Whole microsomes.
2. Rough endoplasmic reticulum.
3. Whole smooth endoplasmic reticulum.
4. Smooth endoplasmic reticulum, subfraction I.
5. Smooth endoplasmic reticulum, subfraction II.
6. 0.01 M EDTA + 0.15 M KCl (in Tris-HCl buffer) wash of microsomes: supernatant fraction.

with Tris-HCl buffer containing 0.01 M EDTA and 0.15 M KCl (Figure 1, tube 6). Even though there was some success in separating SER and RER as judged by the electrophoresis and quantitation studies of the fractions, the yield of SER obtained by this method was so low that other methods of membrane purification had to be investigated.

Tertiary-amyl alcohol treatment of microsomes.--Treatment of microsomes with tert-amyl alcohol according to the procedure of MacLennan et al. (31) has been shown to yield a membrane fraction essentially free of ribosomes and rich in the electron transfer enzymes of microsomes. The method was applied here in an attempt to obtain a reasonably pure membrane fraction free of major contaminants such as ribosomes and other protein species loosely bound to the membrane. As used here, however, the method only yielded four somewhat crosscontaminated fractions, judging from the close similarities in the RNA-ribose and lipid phosphate contents of each. These fractions (a clear supernatant, a loose reddish pellet, a mixture of loose reddish and brownish pellets, and a light brownish pellet) were observed also to have very similar electrophoresis profiles, which would indicate very little resolution of microsomal membrane components.

Microsome Extraction Procedures

Organic solvent extractions.--In view of the difficulties experienced in attempts to achieve a sufficient

separation and quantity of the membranes of microsomes, methods, which would specifically extract all non-membrane forming proteins (e.g. ribosomal proteins and other proteins loosely bound to the membrane), were investigated. One method attempted was to remove electrostatically bound protein by successive washings of microsomes with pyrophosphate buffers (0.1 M and 0.02 M, pH 7.8) and to resolve the remaining membrane proteins, interacting primarily through hydrophobic bonds (see Literature Review), by organic solvent extractions. The pyrophosphate buffer washed microsomes were lyophilized and 5 mg extracted with the following solvents: glycerol-butanol (1:4 v/v), butanol, dioxane-water (1:4 v/v), dioxane-water (4:1 v/v), pyridine, N,N'-dimethylformamide, N,N'-dimethylformamide-water (1:1 v/v), and 2-aminoethanol. Extraction of the protein samples was performed according to the procedure outlined in Methods and Materials. Of the solvents mentioned above, it was observed that the dioxane-water (4:1 v/v) gave the best results with respect to protein solubilization. A rough estimate as to the extent of protein solubilization indicated that this solvent extracts 2 to 4 times the protein as did the other solvent mixtures.

The effect of divalent cations, for example Mg^{++}, on the solubilization of membrane proteins by some of the organic solvents mentioned above was also investigated. This investigation was initiated due to the findings of

Byington et al. (55) that various aliphatic alcohols, e.g. methyl, ethyl, n-butyl, n-amyl, and n-decyl alcohols, solubilized more mitochondrial membrane proteins in the presence of Mg^{++}. The solvent mixtures used here are shown in Table 2.

TABLE 2.--Solubilization of beef liver microsomal protein by organic solvents in the presence of 0.1 M $MgCl_2$. [Beef liver microsomes isolated according to Method 1, were washed with pyrophosphate buffer, lyophilized, and 0.1 gram samples extracted with organic solvent solutions in the presence and absence of 0.1 M $MgCl_2$ using methods described in Methods and Materials].

Solvent Mixture	% Protein Solubilized[a]
Dioxane:H_2O (2:8)	72.3
Dioxane:H_2O (2.8), 0.1 M in $MgCl_2$	76.0
Dioxane:H_2O (8.2)	87.4
Dioxane:H_2O (8.2), 0.1 M in $MgCl_2$	88.8
N,N[1]-dimethyl formamide (1:1)	71.5
N,N[1]-dimethyl formamide (1:1) 0.1 M in $MgCl_2$	79.5

[a]Expressed as mg protein, as determined by the method of Lowry, in the extracted pellet divided by the weight of the lyophilized membrane preparation.

It is interesting that in each of the solvents used an enhancement in protein solubilization was observed in the presence of Mg^{++}. However, due to the fact that organic solvent treatments have been demonstrated to be

harsh on some proteins, milder methods of membrane resolution were pursued.

Lysis of rat liver microsomes.--Lysis or osmotic shocking of microsomes was a method used to release the soluble contents of microsomal vesicles. It was noted that dilution of microsomes, stored in Tris-HCl buffer containing 50% glycerol, with distilled water resulted in a time dependent clarification. Quantitation studies showed solubilization of 6 to 14% more protein by this procedure than when microsomes were stored in 0.25 M sucrose. The effect of glycerol, present at final concentration of approximately 0.3 M, on the extent of lysis of microsomal vesicles at room temperature was followed with time by light scattering measured at 600 millimicrons according to procedure described in Methods and Materials. Lysis was shown to be completed, both in microsomes lysed in the presence of 2.76% glycerol and those lysed with only distilled water, after approximately 15 minutes using the fact that no further decreases in optical density after this period of time as criteria.

The lysis procedure was also observed to be temperature dependent. It was shown that 7 to 11% more protein could be solubilized by lysis at room temperature and that the presence of 2.76% glycerol enhanced protein solubilization during lysis at both ice bath and room temperatures.

Sonic oscillation of beef liver microsomes.--Beef liver microsomes, isolated according to Method 1 and diluted 1 to 4 with 1.15% KCl, were sonicated for various time periods to determine the extent to which microsomal vesicles were ruptured and their soluble contents released. Microsomes were sonicated, see procedure in Methods and Materials, for periods of 0, 10, 40, 60, 120, and 480 seconds. There was no significant amount of protein solubilized over the control after 480 seconds of sonication (Table 3). Electrophoresis profiles of the proteins of the pellets and supernatants, resulting after centrifugation of the sonicated microsomes, were observed to show little difference from those of the control. It is significant that sonication of microsomes, a procedure which should effectively rupture all microsomal vesicles, did not extract more proteins than did the lysis procedure. This indicates that the lysis procedure (Method 2) was effective in completely rupturing microsomal vesicles.

Acetic acid (1.4% v/v) extraction.--Extraction of microsomes with 1.4% acetic acid was one method thought to be potentially useful as a means of extracting proteins non-essential to membrane structure. This extraction procedure was used successfully by Zahler et al. (44) to extract a protein fraction from mitochondria which was electrophoretically identical to that of structural protein, as defined by Green et al. (10). Also, Zahler was

able to show by electron microscopy that this extraction procedure did not destroy membrane structure. In this investigation liver microsomes from phenobarbital treated rats were lysed, according to Method 2, extracted twice with 1.4% acetic acid, and the polyacrylamide gel electrophoresis protein profile of the various fractions determined (Figure 2). Judging from the electrophoresis patterns of the extracted pellet and proteins precipitated from the supernatant by raising the pH to 6.5 (Figure 2, tubes 2 and 3) this treatment does not extract the protein band designated as structural protein in this study. It can be seen in Table 4 that the protein extracted by this method represents approximately 40% of membrane protein, a property which for reasons previously mentioned in the Introduction and Literature Review may justify assigning to it a structural function. However, the fact that it does not appear to contain any appreciable amount of phospholipid, as inferred by the phospholipid content of membrane proteins before and after the acetic acid extraction (Table 4), would tend to rule out such a function for this extractable protein fraction.

It must be mentioned that the pH of the 1.4% acetic acid solution was around 3.1, a condition known to denature most proteins. Also the fact that proteins were visually seen to coagulate and precipitate out of the microsomal suspension under these conditions gave further reason to abandon this treatment as a means of

Fig. 2.--EDTA-KCl and 1.4% acetic acid extractions of rat liver microsomes.

1. Lysed microsomal pellet.

2. Lysed + 1.4% acetic acid twice extracted pellet.

3. 1.4% acetic acid extraction supernatant; proteins precipitated by raising pH to 6.5.

4. Lysed + 0.01 M EDTA, 0.15 M KCl (in Tris-HCl buffer) twice extracted.

5. 0.01 M EDTA, 0.15 M KCl extraction supernatant.

TABLE 3.--Quantitation of the effects of sonication on beef liver microsomal protein solubilization. [Beef liver microsomes, isolated according to Method 1 (see Methods and Materials) and stored in Tris-HCl buffer, 50% in glycerol, were thawed and diluted 1 to 4 with 1.15% KCl before being used in this experiment].

Time Sonicated (Total Seconds)	% Protein Solubilized[a]
0	11.5-22.3
10	10.5-11.4
40	10.3-19.7
60	11.4-30.5
120	12.4-18.3
480	13.0-18.4

[a]Due to the difficulties incurred in the quantitation of protein solubilized, upper and lower limits of the per cent solubilization were set according to quantitation of the protein contents of the pellets and supernatants, respectively, after centrifugation.

TABLE 4.--Analyses of acetic acid and EDTA extracted rat liver microsomal membranes. [Proliferation of liver endoplasmic reticulum was induced by phenobarbital treatment. Microsomes were lysed according to Method 2 and samples were washed twice with either acetic acid or EDTA solution].

Sample Treatment	Total Phosphate ($\frac{\mu mole}{mg\ protein}$)	Lipid Phosphate ($\frac{\mu mole}{mg\ protein}$)	Ribose ($\frac{mg \times 10^3}{mg\ protein}$)	% Protein Solubilized
Untreated microsomes	5.43	3.08	27.3	----
Lysed microsomes	5.27	3.34	19.3	24.9
Lysis + 1.4% acetic acid extraction	7.06	3.91	20.55	40.0
Lysis + 10 mM EDTA, 0.15 M KCl, Tris-HCl (0.05 M, pH 7.5) extraction	4.83	3.47	12.55	29.1

obtaining desirable membrane preparation with which to work.

Salt extractions of beef and rat liver microsomes.-- Since the previous methods of resolving microsomal membrane proteins resulted in varying degrees of cross contamination and/or possible denaturation of the different protein fractions obtained, methods were sought which would specifically extract proteins from microsomal membranes. Extraction of microsomes with various salts in Tris-HCl buffer were performed in an attempt to separate ribosomes and other readily detachable proteins from the lipoprotein network believed to be the basis of membrane structure (3, 4). The extent to which this was accomplished was determined by monitoring RNA-ribose and lipid-phosphate content of the various protein fractions. Of the several salts used initially, Table 5, KSCN was shown to best solubilize non-lipoprotein whereas NaBr and KNO_3 were most effective in extracting RNA-ribose. Higher concentrations, 2.0 M, of salts used for the extractions (Table 6) gave similar results with respect to the effectiveness with which the various salts used solubilized non-lipoprotein and RNA-ribose.

Electrophoresis profiles of beef liver microsomal membranes extracted with various salts (Figure 3) show close similarities with respect to the protein species solubilized. It was also observed that most of these

TABLE 5.--Analyses of beef liver microsomes following extractions with various salts. [Microsomes, isolated according to Method 1 in Methods section, were lysed according to lysis Method 1. All samples were extracted twice, once with 0.5 M followed by 1.0 M salt concentrations].

Sample Treatment	Total Phosphate[c] ($\frac{\mu moles}{mg\ protein}$)	Lipid Phosphate ($\frac{\mu moles}{mg\ protein}$)	Ribose ($\frac{mg \times 10^3}{mg\ protein}$)
Lysed microsomes	4.23	1.88	3.59
Control pellet[a]	3.12	2.09	1.67
$MgCl_2$ extractions	3.84	4.86	4.73
NaBr extractions	3.52	4.45	0.575
KSCN extractions	5.54	7.20	3.22
KNO_3 extractions	4.00	4.18	0.388
Urea extractions[b]	3.98	5.20	2.04
$CaCl_2$ extractions	2.96	4.17	3.80

[a]Lysed microsomal sample was extracted twice with 0.05 M Tris-HCl, pH 7.5 buffer.

[b]The first extraction was performed in 1.0 M urea and the second extraction with 2.0 M urea.

[c]Total phosphate salt extracted samples is in error in that the lipid content of samples was underestimated due to the presence of relatively high salt concentrations.

TABLE 6.--Analyses of beef liver microsomal membranes extracted with high concentrations of various salts. [Microsomes were lysed according to Method 1 in Methods and Materials section, all samples extracted once].

Sample Treatment	Total Phosphate ($\frac{\mu mole}{mg\ protein}$)	Lipid Phosphate ($\frac{\mu mole}{mg\ protein}$)	Ribose ($\frac{mg \times 10^3}{mg\ protein}$)	% Protein Solubilized by Treatment
Lysed microsomes	3.96	2.04	2.62	----
Control pellet[a]	3.55	3.14	2.13	22.8
2.0 M NaBr extraction	7.12	4.03	1.14	27.7[b]
2.0 M KSCN extraction	6.65	6.30	2.07	38.6[b]
2.0 M KNO_3 extraction	5.80	3.15	1.85	11.7[b]
2.0 M NaCl extraction	5.31	3.50	1.58	13.5[b]

[a]Lysed microsomes were extracted once with 0.05 M Tris-HCl, pH 7.5, buffer.
[b]Corrected for protein solubilized by the control.

salt-labile proteins were electrophoretically identical to the proteins solubilized by lysis of beef liver microsomes in 2.76% glycerol. This observation generally holds true for most salts, containing monovalent anions (NaBr, KSCN, KNO_3, NaCl), investigated. One exception was found when 2.0 M KSCN was used to extract microsomes. This salt was shown to effectively solubilize all but two protein components of microsomal membranes (Figures 3 and 4). The protein band with the slower electrophoretic mobility, because of the fact that it is identical to the predominating protein species of whole rat and beef liver microsomes (compare tubes 1 and 6 in both Figures 3 and 4), has been designated in these studies as being a structural protein of microsomal membranes. Though a structural role for this protein band has not been demonstrated, further evidence have been presented which may suggest its function. The protein fraction not extractable with KSCN has a relatively high lipid content compared to whole microsomes, see Tables 5, 6, and 7. This property would suggest that this protein is one of the proteins in the lipoprotein repeating units thought to be the basis of membrane structure (3, 4).

The proteins extracted from rat liver microsomes, isolated from rats treated with phenobarbital, with salts containing polyvalent anions (e.g. citrate, phosphate, pyrophosphate, and carbonate) are shown in Figure 5.

Fig. 3.--Salt extractions of beef liver microsomes.

1. Lysed microsomal pellet.
2. Lysed + Tris-HCl extraction (control) pellet.
3. Lysed + Tris-HCl extraction supernatant.
4. Lysed 2.0 M NaBr extracted pellet.
5. Lysed 2.0 M NaBr extracted supernatant.
6. Lysed 2.0 M KSCN extracted pellet.
7. Lysed 2.0 M KSCN extracted supernatant.
8. Lysed 2.0 M KNO_3 extracted pellet.
9. Lysed 2.0 M KNO_3 extracted supernatant.
10. Lysed 2.0 M NaCl extracted pellet.
11. Lysed 2.0 M NaCl extracted supernatant.
12. SP isolated from beef liver microsomes by detergent treatment.

Fig. 4.--Effect of increasing salt concentrations on the extraction of proteins from microsomes of phenobarbital treated rats.

1. Untreated microsomes.
2. Tris-HCl washed microsomes (control).
3. 0.5 M NaBr extracted pellet.
4. 1.5 M NaBr extracted pellet.
5. 0.5 M KSCN extracted pellet.
6. 1.0 M KSCN extracted pellet.
7. 1.5 M KSCN extracted pellet.

Fig. 5.--Extraction of liver microsomes from control rats with salts containing polyvalent anions.

1. Lysis + 0.5 M sodium citrate extraction supernatant.
2. Lysis + 0.1 M sodium phosphate extraction supernatant.
3. Lysis + 0.1 M sodium pyrophosphate extraction supernatant.
4. Lysis + 0.05 M sodium carbonate extraction supernatant.
5. Lysis + 0.5 M sodium bromide extraction supernatant.
6. Lysis + Tris-HCl extraction supernatant.

TABLE 7.--Analysis of rat liver microsomal membranes extracted with various concentrations of salts. [Proliferation of liver ER was induced by phenobarbital treatment of rats according to Methods and Materials section. Microsomes were isolated according to Method 1 but were stored in Tris-HCl buffer, 50% in glycerol for Experiment 1 and in 0.25 M sucrose for Experiment 2. In the case of Experiment 3 microsomes are isolated according to Method 2 and are stored in Tris-HCl buffer at 0-4° and used within 8 hours after isolation. Lysis of microsomes was performed according to Method 1 for Experiment 1 and Method 2 for Experiments 2 and 3].

Experiment Number	Treatment of Sample	Total Phosphate ($\frac{\mu mole}{mg\ protein}$)	Lipid Phosphate ($\frac{\mu mole}{mg\ protein}$)	RNA-ribose ($\frac{mg \times 10^3}{mg\ protein}$)	% Protein Solubilized
1	Lysed microsomes	3.73	2.57	18.1	----
1	Control pellet[a]	3.91	3.25	15.3	10.8
1	0.05 M NaBr extraction	4.22	3.36	16.3	7.2[b]
1	0.25 M NaBr extraction	4.35	3.65	16.1	10.2[b]
1	0.50 M NaBr extraction	5.00	4.02	16.8	19.4[b]
1	1.00 M NaBr extraction	5.49	4.19	18.9	22.8[b]
1	1.50 M NaBr extraction	6.32	4.23	18.2	23.2[b]
1	1.0 M KSCN extraction	8.94	7.10	34.2	47.5[b]
1	1.5 M KSCN extraction	9.88	8.60	41.4	55.6[b]
1	2.0 M KSCN extraction	10.45	10.22	48.1	60.8[b]
1	0.5 M KNO_3	4.35	4.05	16.8	16.2[b]
1	1.0 M KNO_3 extraction	3.85	3.51	16.5	15.8[b]
1	1.5 M KNO_3 extraction	4.47	3.80	17.1	17.5[b]
2	Untreated microsomes	5.43	3.08	2.73	----
2	Lysed microsomes	5.27	3.34	19.3	24.9
2	Control pellet	6.27	3.55	19.4	19.1
2	0.05 M LiCl extraction	5.75	3.57	17.5	----
2	0.50 M LiCl extraction	6.25	3.63	15.4	1.40[b]
2	0.70 M LiCl extraction	----	----	----	8.10[b]
2	1.00 M LiCl extraction	5.78	3.85	19.5	5.40[b]
2	1.50 M LiCl extraction	5.80	3.94	21.0	9.10[b]
2	2.00 M LiCl extraction	6.20	3.81	20.8	7.70[b]
2	2.50 M LiCl extraction	5.97	4.03	21.7	13.9[b]
3	Unfrozen, lysed microsomes	----	2.75	14.9	7.7[c]
3	Control[a]	----	2.40	16.9	7.29[c]
3	0.10 M $Na_2C_6H_5O_7$ extraction	----	2.29	10.6	~0[c]
3	0.50 M $Na_2C_6H_5O_7$ extraction	----	2.88	13.3	12.2[c]
3	0.05 M Na_2HPO_4 extraction	----	2.15	10.5	1.64[c]
3	0.10 M Na_2HPO_4 extraction	----	2.16	12.1	1.51[c]
3	0.30 M Na_2HPO_4 extraction	----	2.74	11.6	3.11[c]
3	0.01 M $Na_4P_2O_7$ extraction	----	3.22	11.6	0.94[c]
3	0.05 M $Na_4P_2O_7$ extraction	----	3.20	12.1	4.25[c]
3	0.10 M $Na_4P_2O_7$ extraction	----	3.42	13.5	5.41[c]
3	0.05 M Na_2CO_3 (pH 10.0 extraction)	----	2.66	9.04	1.07[c]
3	0.50 M $NaNO_2$ extraction	----	2.95	18.6	----
3	0.50 M NaBr extraction	----	2.57	16.2	6.74[c]

[a]Control, lysed microsomes extracted once with 0.05 M Tris-HCl, pH 7.5, buffer.

[b]Corrected for protein solubilized by control.

[c]Protein determined on supernatant fractions by determining their optical densities at 280 and 260 millimicrons, corrections made for control solubilization when necessary; the effects of salts or salt concentrations on optical density readings at 280 and 260 mμ are not known.

The concentrations of these salts were kept relatively low, from 0.01 M to 0.50 M, because of their solubility properties under the conditions used in these experiments. For comparison, a tube showing the electrophoresis profile of proteins extracted with 0.5 M NaBr is included. Some differences can be seen with respect to the amounts of the faster migrating proteins extracted with these salts when compared to those proteins typically extracted with salts containing monovalent anions such as NaBr. It was also observed in other experiments that some of these faster migrating proteins were identical to those extractable with Tris-HCl buffer containing 0.01 M EDTA and 0.15 M KCl (Figure 2, tube 5). With the exception of these differences, the electrophoretic profiles of the proteins extractable by these salts are very similar with respect to the specific proteins extracted and only minor differences are seen in relation to the amounts of proteins extractable (Figure 5).

The effects of varying salt concentrations on the extent of protein and RNA-ribose extraction from liver microsomes of phenobarbital treated rats were also investigated (Table 7). Optimum concentrations for maximum solubilization of non-lipoprotein seems to be around 1.5 M for NaBr, 2.0 M for KSCN, 1.5 M for KNO_3 and 2.5 M for LiCl. The other salts tested showed increase in protein extraction with salt concentrations; however, due to the

limited solubilities of salts such as sodium citrate, phosphate, and pyrophosphate an optimum, as such, was not determined. Although KSCN solubilizes more protein than any of the other salts used, the evidence presented in Table 7 indicates that ribosomes are not being extracted from microsomal membranes, the criteria being the relatively high RNA-ribose content of the extracted microsomal proteins.

Figure 4 shows the effects of increasing salt concentrations on the extent to which proteins were extracted from microsomal membranes isolated from phenobarbital treated rats. Only KSCN showed a correlation between salt concentration used and the specificity of the proteins so removed from the electrophoresis profiles of the extracted microsomes. Extractions with various concentrations of other salts, for example NaBr shown in this figure, showed no such correlation.

EDTA-KCl extraction of microsomes.--Ribosomes have been generally thought to be attached to the endoplasmic reticulum (ER) by a combination of electrostatic binding and magnesium complexing. It is reasonable, therefore, to expect that chemical agents which are known to complex with or remove magnesium and weaken electrostatic bonds should detach ribosomes from ER membranes. Following the above reasoning, the effects of washing microsomes with solutions containing a known magnesium complexing agent, EDTA, in the presence of 0.15 M KCl was investigated.

Liver microsomes from phenobarbital treated rats, isolated according to Method 2 in the Methods and Materials section, were thawed and washed by diluting aliquots in 40 ml centrifuge tubes to a final protein concentration of about 2.5 mg/ml with the desired buffers (Table 6) and mixing the contents by inverting the tubes several times. The tube containing the unbuffered EDTA-KCl solution (pH 4.9) coagulated the proteins and had to be mixed by homogenizing in a Potter-Elvehjem tissue homogenizer, fitted with a teflon pestle, for one minute at 0-4°. The tubes were then left in an ice bath for 20 minutes and then centrifuged for 100 minutes at 105,000g. The pellets were analyzed for RNA-ribose and lipid-phosphate. Supernatants were dialysed against distilled water for about 16 hours and lyophilized before electrophoresis was performed. Of the treatments shown in Table 8, washing microsomes with Tris-HCl buffer containing 0.01 M EDTA and 0.15 M KCl at pH 7.5 was most effective in extracting ribosomes and/or non-lipoproteins. The criteria used in making this observation is the relatively low RNA-ribose content coupled with an increase in the lipid-phosphate of the extracted pellet in comparison to those values observed in the control and the other extracted pellets.

The electrophoresis profiles of the extracted microsomal fractions and their supernatants are shown in Figure 6. It is interesting that each treatment removed one of

TABLE 8.--Analysis of Tris-HCl buffer, KCl, and EDTA extracted rat liver microsomal membranes. [Proliferation of the endoplasmic reticulum was induced by phenobarbital treatment].

Treatment of Samples	Lipid Phosphate ($\frac{\mu mole}{mg\ protein}$)	Ribose ($\frac{mg \times 10^3}{mg\ protein}$)	% Protein Solubilized
Untreated microsomes	2.14	14.67	----
0.05 M Tris-HCl, pH 7.5 extraction	3.31	18.55	28.8
0.05 M Tris-HCl, pH 7.5 + 0.15 M KCl extraction	4.48	25.87	52.4
0.05 M Tris-HCl, pH 7.5 + 10 mM EDTA extraction	4.06	19.55	42.8
10 mM EDTA + 0.15 M KCl, pH 4.9 extraction	3.84	25.30	46.4

Fig. 6.--EDTA-KCl wash of rat liver microsomes (phenobarbital treated rats).

1. Untreated microsomes.
2. Tris-HCl washed pellet.
3. Tris-HCl washed supernatant.
4. Tris-HCl + 0.01 M EDTA + 0.15 M KCl washed pellet.
5. Tris-HCl + 0.01 M EDTA + 0.15 M KCl washed supernatant.
6. 0.01 M EDTA + 0.15 M KCl (pH 4.9) washed pellet.
7. 0.01 M EDTA + 0.15 M KCl (pH 4.9) washed supernatant.
8. Tris-HCl + 0.15 M KCl washed pellet.
9. Tris-HCl + 0.15 M KCl washed supernatant.
10. Lysis supernatant.
11. Crude ribosome preparation.

the more electrophoretically mobile proteins associated with a densely stained protein-band of a crude rat liver ribosome preparation. This particular band is not removed by lysis of microsomal vesicles, indicating that it is not due to one of the soluble proteins located within microsomal vesicles. Also by comparing the electrophoresis profiles of the proteins solubilized with 0.15 M KCl in Tris-HCl buffer (compare tubes 5 and 9 with tube 3, Figure 6) one can again see that those proteins which are salt labile are also labile to lysis.

In Figure 2, tubes 4 and 5 show the extracted protein pellet and supernatant protein fraction of rat liver microsomes which were lysed, according to Method 2, and washed twice as mentioned above with Tris-HCl buffer containing 0.01 M EDTA and 0.15 M KCl at pH 7.5. This figure shows more clearly that the EDTA-KCl washing specifically extracts proteins of greater electrophoretic mobility than those associated with the lysis supernatant shown in Figure 6. Quantitative studies of the extracted pellet (Table 4) show a relatively low RNA-ribose content, suggesting that the proteins extracted from the lysed microsomes, are ribosomal. The electrophoretic profiles, shown in Figure 6, would thus indicate a possible removal of microsomal vesicle content as well as ribosomal proteins by washing microsomes with Tris-HCl buffer containing EDTA and KCl.

Resolution of Rat Liver Microsomal Membrane Proteins by Combinations of Treatments

Microsomes from the livers of phenobarbital treated rats were extracted successively by the treatments listed in Table 9. The protein pellets from the various treatments were collected by centrifugation at 105,000g for 100 minutes in the case of lysis and the EDTA-KCl washes and for 2 hr after the salt extractions. The pellets were then analyzed for their protein, RNA-ribose, lipid and total-phosphate contents. Lysis of microsomes was performed according to Method 2 and the salt extractions and EDTA-KCl washes according to Methods and Materials and the Results section, respectively. Lysis of microsomes extracted some lipid as well as RNA-ribose.

Washing with EDTA-KCl in experiment 1 (Table 9) extracts a significant amount of RNA-ribose, an observation not in accord with the results shown for experiment 2 where most of the readily extractable RNA-ribose is removed by lysing microsomes. One interesting observation is that KSCN extraction removes RNA-ribose only when this treatment is preceded by NaBr extraction. This fact is also contrary to the results shown in Tables 5-7 regarding the effects of KSCN treatment alone on microsomes. According to these data KSCN extraction does not remove RNA-ribose but in fact concentrate it with respect to the control and some of the other salt extracted pellets. It has also been observed that the electrophoresis profile of the protein

TABLE 9.--Analysis of rat liver microsomes during membrane purifications. [Livers from rats treated with phenobarbital were lysed according to Method 2].

Experiment Number	Treatment[a]	Total Phosphate ($\frac{\mu mole}{mg\ protein}$)	Lipid-Phosphate ($\frac{\mu mole}{mg\ protein}$)	RNA-ribose ($\frac{mg \times 10^3}{mg\ protein}$)	% Protein Solubilized by Each Treatment	% Protein Solubilized by Combined Treatment
1	Untreated microsomes	6.25	3.54	23.2	----	----
1	Lysis (1)	5.87	3.26	19.5	5.70	5.70
1	EDTA-KCl extraction (2)	7.33	3.84	13.3	9.20	14.90
1	1.5 M NaBr extraction (3)	7.81	5.85	13.5	38.4	53.3
1	2.0 M KSCN extraction (4)	7.51	9.92	4.3	29.9	83.2
2	Untreated microsomes	4.37	1.92	18.1	----	----
2	Lysis (1)	4.16	1.57	11.3	----	----
2	EDTA-KCl twice washed (2 and 3)	4.64	3.01	10.8	43.9	43.9
2	1.0 M KSCN extraction (4)	9.26	6.13	11.3	49.6	93.4

[a]Treatments in each experiment are listed in the order in which they were performed.

pellets resulting from extractions of microsomes with 2.0 M KSCN alone and with the combination of treatments in experiment 1, Table 9, are essentially identical. This latter finding raises the question of whether or not RNA is being extracted alone or as a complex with ribosomal proteins.

Isolation of Structural Protein from Control and Phenobarbital Treated Rats

A crude structural protein fraction, as defined by Green et al. (10), was isolated from the livers of control and phenobarbital treated rats. The electrophoresis profiles of both control and phenobarbital treated microsomes and the crude structural protein fraction isolated from each are shown in Figure 7. It can be seen that phenobarbital treatment of rats induces a specific group of proteins in the electrophoretic profile. The fact that phenobarbital treatment is known to cause a proliferation of SER of the liver (56) and that these induced proteins appear to be identical to those representing the predominate protein species of control microsomes, would suggest a structural function for these proteins. Keeping in mind the facts that membranes are currently thought to be expressions of lipoprotein subunits (3, 4) and that the proteins not extracted by 2.0 M KSCN, one of which is identical to the "induced" microsomal proteins (Figure 7, tube 3), have a relatively high lipid content, Tables 5-7, one can see further reasons for suggesting a structural role for these proteins.

Fig. 7.--Isolation of SP from control and phenobarbital induced rat liver microsomes.

1. Phenobarbital induced microsomes.
2. Phenobarbital induced microsomal SP.
3. 2.0 M KSCN extracted microsomal pellet.
4. Control microsomes.
5. Control microsomal SP.

It has been observed in this and other experiments, however, that in crude structural protein isolated from control microsomes by detergent treatment the predominate protein species shown in the electrophoresis profiles (Figure 7, tube 5) is not the one induced by phenobarbital treatment; neither has it been observed to be the predominate protein species of control microsomes (Figure 7, tube 4). In light of these data, structural protein as defined by Green et al. (10) warrants re-evaluation.

Effects of Various Treatments and Extraction Procedures on the Enzymatic Activities of Microsomes

Effects of freezing and lysis on NADPH-cytochrome c reductase and Aminopyrine demethylase activity of microsomes.--A meaningful study of membrane proteins requires relatively mild methods of resolution which would yield proteins in a state analogous to that in vivo. Keeping this in mind the effects of the various treatments, used in these resolution studies on microsomal membranes, on enzymatic activities of microsomes were determined. For these studies enzymatic activities closely associated with the microsomal membrane, i.e., NADPH-cytochrome c reductase "reductase" and Aminopyrine demethylase "demethylase," were monitored and used as a gauge in determining the harshness of the various treatments. Aminopyrine demethylase activity was measured at two or more substrate concentrations. This was done in light of evidence obtained for

there being two or more enzymatic components responsible for demethylation of aminopyrine, i.e., non-linear Michaelis Menton kinetics (73).

The first of these treatments investigated were those of freezing and lysis of microsomes. The data showing the effects of these treatments on enzymatic activities of microsomes is summarized in Table 10. It is seen that suspending microsomes, freshly isolated from the livers of rats treated with phenobarbital, in Tris-HCl buffer (50% glycerol) or in 0.25 M sucrose increased both the reductase and demethylase activities. Freezing microsomes in Tris-HCl buffer (50% glycerol) did not change reductase activity but destroyed from 3 to 15% of the demethylase activity at different substrate levels. Freezing in 0.25 M sucrose, however, caused a loss in both activities. The increased activities observed by lysis of the control, Tris-HCl buffer (50% glycerol) and 0.25 M sucrose frozen microsomes, probably reflect differences in solubilization of microsomal membrane proteins (see Discussion under Lysis of Microsomes) rather than an activation of these enzymes.

EDTA-KCl wash: Effects on enzymatic activities of microsomes.--The observation made earlier that washing with Tris-HCl buffer (0.01 M EDTA and 0.15 M KCl) extracted proteins assumed to be ribosomal (Figures 1 and 2), led to the investigation of its effects on enzymatic activity. The data summarized in Table 11 show decreases in NADPH-cytochrome c reductase and aminopyrine demethylase activity

TABLE 10.--Effects of freezing and lysis on the activities of microsomal enzymes. [Microsomes from the livers of rats treated with phenobarbital were used in these studies. Freezing of microsomes was achieved by storage at -15° for 2 days and lysis of all samples was performed according to Method 2 (Methods and Materials). Control microsomes were those stored in Tris-HCl buffer at 4° and used a few hours after isolation. Aminopyrine substrate levels represent that in the incubation (Methods and Materials)].

Sample Treatment	NADPH-cytochrome c Reductase Activity (% specific activity of control)	Aminopyrine Demethylase Activity (% specific activity of control)	
		4.0×10^{-3} M Aminopyrine	4.0×10^{-4} M Aminopyrine
Microsomes suspended in Tris-HCl, 50% glycerol	117.4	153.0	164.0
Microsomes suspended in 0.25 M sucrose	118.8	143.0	174.2
Freezing in Tris-HCl, 50% glycerol	115.8	85.4	97.2
Freezing in 0.25 M sucrose	81.2	81.0	95.3
Lysis of control microsomes	116.3	101.5	115.7
Lysis of Tris-HCl, 50% glycerol frozen microsomes	115.7	106.0	127.6
Lysis of 0.25 M sucrose frozen microsomes	97.5	107.5	140.0

TABLE 11.--The effects of EDTA-KCl washing on microsomal enzymes. [Microsomes used were those isolated from control male rats according to Method 2 (Methods and Materials). Washing was performed by suspending microsomes, stored at -15° in Tris-HCl (50% glycerol), in the appropriate buffers, incubating for 30 minutes at 4°, centrifugation at 105,000g for 90 minutes, and resuspended in Tris-HCl buffer. Control microsomes were those stored in Tris-HCl (50% glycerol) which underwent no further treatment. Aminopyrine levels represent those in the incubation mixture (Methods and Materials)].

Buffers Used to Wash Microsomes	NADPH-cytochrome c Reductase Activity (% specific activity of control)	Aminopyrine Demethylase Activity (% specific activity of control)	
		4.0×10^{-3} M Aminopyrine	4.0×10^{-4} M Aminopyrine
Tris-HCl	92.4	62.4	28.0
Tris-HCl + 0.01 M EDTA	104.1	125.7	99.4
Tris-HCl + 0.15 M KCl	76.5	62.4	44.6
Tris-HCl + 0.01 M EDTA + 0.15 M KCl	100.5	118.3	83.4

resulting from washing microsomes with Tris-HCl buffer and Tris-HCl buffer, 0.15 M in KCl. The increased inhibition of reductase activity observed by the latter may be interpreted as a decrease in the protein extraction by the presence of KCl. It must be stressed here that washing microsomes with 0.15 M KCl was reported not to inhibit or extract NADPH-cytochrome c reductase (52); interpretations of this data should bear this fact in mind.

The apparent increase in reductase and demethylase activities resulting from washing microsomes with Tris-HCl buffer (0.01 M EDTA) and Tris-HCl buffer (0.01 M EDTA + 0.15 M KCl) may reflect an extraction of proteins, e.g. ribosomal, not associated with these activities. It must be mentioned here that in interpreting these data one should bear in mind the fact that they represent differences in specific activities of the extracted pellets as compared to the control, Table 11, and that these treatments were found to extract different protein species (Figure 1, tube 6 and Figure 5, tube 6).

Salt extractions: Effects on enzymatic activities of microsomes.--High concentrations of salts, especially those containing monovalent anions, were found effective in extracting microsomal membrane proteins (Tables 5-7). The effects these salts have on reductase and demethylase activities were determined in order to evaluate the mildness of these treatments. These activities were determined

from microsomes "incubated" and "extracted" with concentrations of the salts listed in Tables 12 and 13. Incubation of microsomes with these salts was performed by suspending aliquots of rat liver microsomes (stored in Tris-HCl buffer, 50% glycerol at -15°) to a concentration of 4.6 to 4.8 mg protein/ml in Tris-HCl buffer containing various concentrations of salts. Aliquots of this was added directly to an assay mixture containing 2.5 μmole NADPH instead of the NADPH-generating system described in Methods and Materials. This was done in order to avoid any effects that these salts might have on isocitrate dehydrogenase, used as part of the NADPH generating system. Extraction of microsomes with salts was performed according to the procedure outlined in Methods and Materials which involved centrifugation of the "incubation" microsomes for 2 hr at 105,000g and resuspension of the pellet in Tris-HCl buffer.

The effects of the salts investigated (K_2HPO_4, NaBr, and NaCl) on reductase and demethylase activities are summarized in Tables 12 and 13. The data shown in Table 12 indicates an activation of NADPH-cytochrome c reductase by salts containing monovalent anions, e.g. NaBr and NaCl, especially at high concentrations (1.0 M). Salts containing polyvalent anions, i.e., K_2HPO_4, had little effect on these activities. Salt extractions were found, generally, not to extract NADPH-cytochrome c reductase as deduced

TABLE 12.--The effects of salts on NADPH-cytochrome c reductase activity. [Microsomes used in these studies were isolated from control rats according to Method 1 (Methods and Materials). Enzymatic activities were determined on microsomes incubated for 1 hr in Tris-HCl buffer containing various levels of salts and either underwent no further treatment (salt incubation) or were centrifuged at 105,000g for 2 hr (salt extraction)].

Salts and Concentrations Used	NADPH-cytochrome c Reductase Activity (% specific activity of control)	
	Salt Incubation[a]	Salt Extraction[b]
0.5 M K_2HPO_4	102.5	116.5
1.0 M K_2HPO_4	100.5	113.7
0.25 M NaBr	102.2	109.4
0.5 M NaBr	104.2	115.6
1.0 M NaBr	120.0	103.5
0.25 M NaCl	111.8	87.2
0.5 M NaCl	114.3	88.0
1.0 M NaCl	120.0	111.6

[a]Control was prepared by dilution of microsomes with Tris-HCl buffer.

[b]Control was prepared by extracting microsomes with Tris-HCl buffer.

TABLE 13.--The effects of salts on aminopyrine demethylase activity. [Microsomes used in these experiments were isolated from control rats according to Method 1 (Methods and Materials). Aminopyrine demethylase activity was determined on microsomes incubated for 1 hr in Tris-HCl buffer containing various levels of salts and either underwent no further treatment (incub.) or were centrifuged at 105,000g for 2 hr (ext'd.). Aminopyrine levels of the incubation mixtures (Methods and Materials) are given].

Salts and Concentrations Used	Aminopyrine Demethylase Activity (% specific activity of control)					
	4.0×10^{-3}M Aminopyrine		1.0×10^{-3}M Aminopyrine		4.0×10^{-4}M Aminopyrine	
	incub.	ext'd.	incub.	ext'd.	incub.	ext'd.
0.10 M K_2PO_4	-----	107.0	-----	131.3	-----	-----
0.50 M K_2PO_4	110.0	121.0	-----	165.3	212.0	134.5
1.00 M K_2PO_4	114.0	111.5	-----	130.3	245.0	-----
0.05 M NaBr	-----	88.8	-----	101.1	-----	-----
0.25 M NaBr	77.9	-----	-----	112.1	145.5	-----
0.50 M NaBr	105.0	50.1	-----	65.1	357.5	65.3
1.00 M NaBr	56.3	34.7	-----	54.1	251.0	-----
0.05 M NaCl	-----	88.6	-----	104.5	-----	-----
0.25 M NaCl	89.4	93.2	-----	95.4	100.0	-----
0.50 M NaCl	91.4	66.2	-----	71.5	227.5	42.7
1.00 M NaCl	66.6	-----	-----	88.6	178.0	-----

Note:
Control used for incubation was prepared by diluting microsomes to desired concentration with Tris-HCl buffer whereas extracted control was prepared by a 105,000g centrifugation of incubation control for 2 hrs.

from the increase in reductase activity as compared to control.

The effects of these salts on aminopyrine demethylase activity is summarized in Table 13. Offhand, these data seem to indicate an inconsistency with respect to effects of the different salts on demethylase activity at various substrate concentrations. However, if one keeps in mind the fact that aminopyrine demethylation does not follow Michaelis-Menton kinetics (73), as mentioned earlier, one may rationalize that these salts do not effect all components of demethylase activity in the same manner. For example, K_2HPO_4 was shown generally to increase demethylase activity, with a greater effect observed at low substrate concentrations. On the other hand, NaBr and NaCl were shown to inhibit demethylase activity at high substrate concentration but increase demethylase activity at low substrate concentrations. The effects of increasing concentration of these salts on demethylase activity are difficult to explain. It appears that the salts containing monovalent anions shows an optimum salt concentration of 0.5 M for demethylase activity, whereas K_2PO_4 shows an increase in activity in the presence of increasing salt concentrations.

DISCUSSION

Fractionation of Microsomes

Fractionation on a discontinuous sucrose gradient in the presence of Cs^+ and Mg^{++} ions.--Rat liver microsomes were fractionated on a discontinuous sucrose density gradient in the presence of Cs^+ and Mg^{++} ions according to the method of Dallner et al. (48). The fractionation achieved resulted from changes in density of the microsomal subfractions due to their ability to specifically bind Mg^{++} or Cs^+, e.g. RER and SER I subfractions bind Cs^+ and Mg^{++} ions, respectively, whereas the SER II subfraction does not. That a rather effective fractionation was achieved by this method is suggested by the quantitation studies, Table 1, which show an approximate four-fold difference in RNA-ribose content of RER over that of whole SER. A further indication of the effectiveness of this fractionation procedure is shown in the electrophoresis profiles of the various fractions, Figure 1. For example, the electrophoresis profile of the RER fraction (Figure 1, tube 2) contains some faster migrating protein bands which are not present in any of the SER fractions; because it is known that only the RER fraction contains ribosomes, these proteins are assumed to be ribosomal. Support is given to this assumption by the fact that

Tris-HCl buffer containing 0.01 M EDTA and 0.15 M KCl, a mixture which should disrupt the forces thought to be responsible for the attachment of ribosomes to RER (i.e., a combination of Mg^{++} complexing and electrostatic binding), extracted some of these proteins (Figure 1, tube 6).

One puzzling observation was made with respect to the electrophoresis profile of SER II (Figure 1, tube 5). There is one protein band, migrating just ahead of SP, which is present in all of the microsomal subfractions but seems to be specifically concentrated in this fraction. No attempts at identification of this band have been made, but judging from the relatively intense reddish color of this fraction one may suggest that it is one of the microsomal cytochromes. The recent findings by Holtzman et al. (57) that SER contains more cytochrome P-450 per milligram of protein than RER, taking into account the ribosomal content of this fraction, may suggest its possible identity. However, it must be mentioned that there was no correlation shown in Holtzman's data indicating the distribution of cytochrome P-450 between the subfractions of SER.

Tertiary-amyl alcohol treatment.--Treatment of microsomes with tert-amyl alcohol was reported by MacLennan et al. (31) to redistribute the lipid in microsomes such that a microsomal electron transport membrane, which apparently has a higher affinity for lipid than the other microsomal components, increases its lipid content. This

membrane fraction becomes less dense and could be separated from the other microsomal components by centrifugation. The method as used here, however, failed to yield the three fractions reported by MacLennan (31). Instead, four fractions resulted one of which appears to be a mixture of two of the other fractions (i.e., the loose reddish pellet, containing the microsomal electron transport membranes, and the tight brownish pellet) indicating incomplete separation. Quantitation of lipid-phosphate and RNA-ribose content of the four fractions obtained also suggest considerable cross-contamination, possibly resulting from incomplete sedimentation of the tert-amyl alcohol treated microsomes.

Extraction Procedures

Organic solvent extraction.--It has been generally accepted that the forces primarily responsible for membrane integrity are hydrophobic. According to the membrane model presented in the Introduction and Literature Review of this thesis, these forces express themselves in the interactions between the lipoprotein subunits of membranes. Attempts were made in these studies to remove weakly bound proteins from beef liver microsomal membranes by successive washings with pyrophosphate buffers (0.1 M and 0.02 M, pH 7.8) and to resolve the unextracted membrane proteins by organic solvent treatments which are known to weaken the hydrophobic bonds (58). Of the organic solvents initially

investigated (glycerol-butanol, 1:4 v/v; butanol; dioxane-water, 1:4 v/v; dioxane-water, 4:1 v/v; pyridine; N,N'-dimethylformamide; N,N'-dimethylformamide-water, 1:1 v/v; and 2-aminoethanol) dioxane-water (4:1 v/v) was found most effective with respect to protein solubilization. Also within this series of organic solvents a rough correlation was observed between the extent of protein solubilization and the dielectric constant of the solvents. For example it was found that the order of decreasing effectiveness of protein solubilization by these solvents was, dioxane-water (4:1 v/v)>2-aminoethanol>N,N'-dimethylformamide.

Divalent cations, e.g. Mg^{++}, were found to enhance the protein solubilization by organic solvents, Table 2. It must be mentioned that the numerical values given for the per cent protein solubilization in this table are only relative and do not represent the exact amount of protein being solubilized (see explanation under Table 2). It is interesting, however, that a similar enhancement in solubilization of beef heart mitochondria treated with various aliphatic alcohols and with compounds of which diethylstilbestrol is a prototype, was observed by Byington et al. (55). These authors observed a divalent cation, e.g. Mg^{++} and Ca^{++}, dependent solubilization of up to 30% of the mitochondrial membrane with diethylstilbestrol. Though similar treatments of microsomal membranes with this compound were investigated in these studies, essentially no such solubilization was observed.

The mechanism by which divalent cations enhance protein solubilization is not clear. Byington et al. (55) suggest that the combined action of diethylstilbestrol and divalent cations effectively weakens mitochondrial membranes by causing changes in the conformations of the lipoprotein subunits. They, however, fail to suggest a specific role for the divalent cations in enhancing proteir. solubilization by diethylstilbestrol or organic solvents. One may postulate a function for these ions if it is taken into account that they (e.g. Mg^{++}, Mn^{++}, Ba^{++}, Ca^{++}) are good protein denaturants (59). These ions could exert their enhancing effect on protein solubilization by denaturing proteins, subsequently exposing their hydrophobic regions to the effects of organic solvents or molecules such as diethylstilbestrol. Though there is a degree of speculation involved in this explanation, it seems reasonable for the data observed.

Lysis of rat liver microsomes.--Lysis or osmotic shocking of microsomes by dilution with distilled water was originally investigated as a method of releasing the soluble contents of microsomal vesicles. It was observed that an increase in the amount of protein solubilized (6 to 14%) resulted from lysis of microsomes stored at -15° in Tris-HCl buffer, 50% in glycerol as compared to those stored in 0.25 M sucrose. This effect was initially contributed solely to the effects of freezing on microsomes. Microsomes stored at -15° in 0.25 M sucrose were

subjected to the formation of ice crystals, a condition not present when microsomes are stored in Tris-HCl buffer (50% in glycerol), which are known to disrupt hydrophobic bonds causing denaturation and/or percipitation of some proteins (60). This effect was later observed to be due to glycerol, in that an increase in protein solubilized by lysis of 0.25 M sucrose stored microsomes in 2.76% glycerol (a concentration which approximates that in the lysis experiments using microsomes stored in Tris-HCl buffer, 50% in glycerol).

The observation was made in a separate experiment that lysis in the presence of 2.76% glycerol of freshly isolated microsomes and of microsomes stored at -15° for two days in Tris-HCl buffer (50% in glycerol) and in 0.25 M sucrose showed a distinct correlation between the storage of microsomes and protein solubilization. Using microsomes isolated and resuspended in Tris-HCl buffer as the control, it was found that lysis of microsomes stored in Tris-HCl buffer (50% in glycerol) solubilized approximately 2% more protein than control upon lysis and that microsomes stored in 0.25 M sucrose solubilized approximately 4% less than control. These observations would tend to confirm the fact that protein denaturation, possibly expressed as percipitation of some proteins, occurs upon freezing microsomes in 0.25 M sucrose and to suggest storage in glycerol at -15° may enhance protein

solubilization. However one must determine whether or not the 2% difference is significant enough to warrant the latter interpretation of this data.

A temperature effect was also observed in the lysis treatment of microsomes. An increase in protein solubilization of 7 to 11% results when microsomes are lysed at room temperature for 30 minutes as compared to lysis at 0-5° for the same time period. In both instances the enhancement of protein solubilization by glycerol was observed. Such effects indicate that lysis is an energy requiring process; however, the specific role of glycerol is not known. One could postulate that it acts to weaken the bonds responsible for the integrity of the membranes of microsomal vesicles, causing a more efficient rupture of these vesicles. If this were so one should expect a shorter time requirement for lysis in the presence of 2.76% glycerol. However, the fact that there were no differences in the time required for lysis of microsomes in the presence of 2.76% glycerol and with distilled water, as determined by light scattering, would argue against this interpretation. Another possibility is that glycerol may not effect the extent to which lysis occurs but that enhancement in protein solubilization is due to the extraction of proteins from the microsomal membranes. Support for this is shown by a comparison of the electrophoresis profiles of proteins

solubilized by lysis (Figure 6, tube 10) with those solubilized by the various salt treatments (see Figure 5 and Figure 3, tubes 3, 5, 7, 9, and 11). It is observed that these treatments solubilize essentially the same protein species, but differ with respect to their effectiveness.

Another observation made was that the proteins solubilized by sonication of beef liver microsomes in the presence of approximately 1.2 M glycerol and 0.12 M KCl had electrophoresis profiles similar to the proteins solubilized by lysis. Also, the extent of protein solubilization resulting from sonication was less than that observed by lysis of microsomes in 2.76% glycerol; that is, if one takes into account the protein solubilized by the control. These data suggest that lysis in the presence of 2.76% glycerol was effective in rupturing microsomal vesicles and that glycerol extracts a limited amount of protein from microsomal membranes.

Acetic acid extraction and structural protein (SP) isolation.--The method of extracting membrane proteins with 1.4% acetic acid (pH approximately 3.1) was investigated here because it was found by Zahler et al. (44) to successfully extract from mitochondrial membranes a protein fraction electrophoretically identical to the SP of Green et al. (10). Since the extraction was achieved without destroying mitochondrial membrane integrity, it was suggested that SP originates from the headpieces (43) observed in the electron micrographs (9) of mitochondrial

membranes. These findings led to the postulation of a membrane model composed of two structural or non-catalytic classes of proteins; i.e., one representing that isolateable with detergents (10, 13) (SP) and another, termed "Core Protein" (CP) (43), located in the basepieces of membranes. It seems reasonable that microsomal membranes may also exhibit a similar structural organization, i.e., a readily detachable component and a basepiece or membrane forming component. Evidence was obtained in these studies which could possibly support this postulate.

Extraction of a protein species, electrophoretically identical to the major protein species of Crude SP isolated from control microsomes with detergents (see Methods and Materials), was achieved by treatment of microsomes with 1.4% acetic acid (Figure 3, tube 3 and 7, tube 5). It was observed that this protein species does not represent SP as defined in these studies; i.e., the major protein species in the electrophoresis profile of whole microsomal membranes. Another observation made was that the electrophoresis profiles of microsomes isolated from the livers of rats treated with phenobarbital showed an increase of SP as defined in these studies, Figure 7. Phenobarbitol causes a proliferation of the smooth endoplasmic reticulum of rat livers (56) and should likewise be expected to cause an increase in the proteins responsible for the structure of membranes; i.e., those which compose the lipoprotein repeating

subunits (3,4) or basepieces of membranes. These observations support the definition of SP in these studies and suggests that its function is analogous to CP of mitochondria. Further support was given for the structural function of the proteins species when it was found to be closely associated with lipid. Extraction of most of the contaminating microsomal proteins with 2.0 M KSCN (Figure 7, tube 3) gives a protein fraction with a lipid content two- to four-fold that of the control, Tables 5-7.

It must be stressed that the exact function of the protein, designated as SP in these studies, has not been conclusively demonstrated neither has it been made clear as to the number of proteins involved in maintaining the structure of microsomal membranes. It is conceivable that a protein may function both enzymatically and structurally; examples of this have been shown with respect to the four electron transport complexes of mitochondria (38, 39) (see also the Literature Review section of this thesis). An example of an enzymatic function for SP, isolated according to Green et al. (10), was demonstrated by Woodward and Munkres (61) that SP from respiratory-deficient mutants of Neurospora differs from that of the wild-type by a single amino acid replacement. Further support for the postulate, that proteins may function both structurally and enzymatically, is given by the data of Ernster and Orrenius (56) which show that proliferation of rat liver SER by

phenobarbitol treatment also induced aminopyrine demethylase, cytochrome P-450, and NADPH-cytochrome c reductase activities. It is possible that the increase in the protein bands seen in the electrophoresis profile of phenobarbitol treated rat liver microsomes (Figure 7, tube 1) may represent an increased synthesis of the enzymes mentioned above. Past failures to demonstrate an enzymatic function for SP isolated by detergents (10, 13) from different membrane systems (e.g. mitochondria, microsomes, chloroplasts, etc.) may reflect denaturation of enzymes by these isolation procedures. The recent findings of Schatz and Saltzgabor (62), that SP isolated from beef heart mitochondria contains a considerable amount of denatured ATPase supports this postulate. These data warrants a review of the identity and function of the proteins previously defined by Green et al. (10) as being SP.

The exact number of proteins involved in the structural integrity of microsomal membranes cannot be clearly pinpointed. Electrophoresis profiles of whole microsomes generally contains several protein bands with electrophoretic mobilities very close to that of SP, Figure 7. It was usually observed that one of these bands, defined as SP in these studies, was more heavily stained than the rest. This observation was made with gels stained both with Coomassie blue and Amido Schwartz; however, visualization of this band was usually better

when Coomassie blue was used to stain the gels. It was impossible to determine from the electrophoresis profiles whether or not these bands represent polymers of SP or species of a class of SP, having very similar properties. The latter has been suggested for SP recently isolated and purified from mitochondrial membranes (17, 43) and it may be possible to extrapolate the concept of a class of SP to other membrane systems.

Salt extractions.--Determination of the exact functions of the proteins associated with the membrane forming subunits, the basepieces free of all readily detachable membrane components, requires a pure membrane preparation. Salt extractions of microsomal membrane proteins were investigated as a relatively mild method of removing proteins loosely attached through electrostatic interactions. A variety of salts was used and their effectiveness in extracting non-lipoprotein, i.e., ribosomal and other proteins not essential to membrane structure, from microsomal membranes was determined, Tables 5-7. It was found, generally, that the extent of protein extraction was not a function of ionic strength but was due to specific properties of the salts used. For example the observation was made that extraction of beef liver microsomal membranes with salts containing monovalent cations (e.g. NaBr, KSCN, KNO_3, and NaCl) extracted essentially the same protein species as did the control, Figure 3; however, the extent to which the species were extracted differed significantly,

Table 6. Quantitation of the per cent protein solubilized and the lipoprotein content of pellets extracted with relatively high salt concentrations, 2.0 M, showed an increasing order of efficiency with respect to non-lipoprotein solubilization with the salts: KNO_3<NaCl<NaBr<KSCN. However, a similar correlation with respect to these salts effectiveness in extracting RNA-ribose was not found, Tables 5-7. Extraction of microsomal membranes with KSCN, for example, always resulted in a protein fraction with a relatively high RNA-ribose content, when compared to the control and other salt extracted membrane preparations. There appears to be an inconsistency with these findings if one takes into account the fact that the 2.0 M KSCN extracted pellet has a total-phosphate to lipid-phosphate ratio close to unity (1.05) whereas extraction with the other salts investigated (Table 6) gave a ratio which differs significantly from unity, e.g. 2.0 M NaBr (1.77), 2.0 M KNO_3 (1.84), and NaCl (1.52). These ratios suggest that in the KSCN extracted pellet essentially all of the phosphate present in the samples is due to phospholipid and that the phosphate due to RNA is not fully accounted for. However, if one makes a rough calculation of the phosphate content of RNA-ribose, one finds that phosphate due to RNA is accounted for in the total-phosphate determinations. For example, assume that the RNA extract (see Methods and Materials) contains completely hydrolysed RNA and that there is a 1:1 mole ratio of phosphate to ribose,

one finds for example that in the pellet extracted with 2.0 M KSCN (Table 6) only 0.0138 μmole PO_4/mg protein accounts for RNA-phosphate. It must be mentioned here that essentially a ten-fold difference between the RNA-ribose content of beef and rat liver microsomes was observed (compare the RNA-ribose content in Tables 5 and 6 with those in Table 7) whereas the total and lipid-phosphate contents are similar. These differences could not be traced to experimental errors but it is believed that the discrepancy may have been caused by the presence of substances in beef liver, e.g. high glycogen content, which may have interfered with the RNA-ribose determination. However, in spite of this discrepancy the calculations regarding the RNA-phosphate content would still hold true.

It is difficult to explain the data mentioned above and that presented in Tables 5-7 in terms of detachment and solubilization of membrane bound ribosomes. That is, one cannot distinguish clearly from these data whether the whole ribosome was detached from the membrane and subsequently solubilized or whether ribosomes remained attached to the membrane or were detached but remained insoluble and sedimented with the protein pellet. Neither can definite conclusions be drawn from the electrophoresis profiles of proteins of the salt extracted pellets and their supernatants (Figures 3 and 4) with respect to identification of proteins associated with ribosomes (see discussion

given under Microsomal Fractionation Procedures and Figure 1). Some conclusions may be reached with regard to these data if one considers the reports (63, 64) indicating that salts containing divalent cations, e.g. $MgCl_2$, protect ribosomal structure whereas salts such as LiCl (2.0 M concentration) were shown to solubilize ribosomal proteins. Quantitative studies (Table 5) of membranes extracted with $MgCl_2$ and $CaCl_2$ show a relatively high RNA-ribose content which would suggest that ribosomes remained intact and sedimented with the insoluble membrane proteins. In like manner one may infer that salts containing monovalent cations, with exception of KSCN, exhibit a limited solubilization of ribosomes.

Salts containing polyvalent anions were investigated to determine whether or not the observed effects of extracting microsomal membranes with monovalent anion containing salts were unique. The sodium salts of citrate, phosphate, pyrophosphate, nitrite, and carbonate were investigated. These were generally observed to be less effective in solubilizing non-lipoprotein but more effective in solubilizing RNA-ribose than monovalent anion containing salts (Table 7, experiment 3). The latter observation suggests solubilization of ribosomes. Some supporting evidence is given in that the electrophoresis profiles of the proteins extracted with these salts contain protein bands associated in these studies with ribosomes (compare the electrophoresis profile of RER in Figure 1 with the profiles shown in Figure 5).

Also it was shown (Figure 5) that in addition to ribosomal proteins these salts solubilize essentially the same protein species as did the control and the salts containing monovalent anions. Therefore, there appear to be definite differences in the proteins extractable with salts containing monovalent and polyvalent anions and the extent to which proteins are solubilized by such treatments is determined by specific properties of these salts rather than ionic strength effects.

These data would suggest that proteins were extracted by mechanisms other than that involving weakening of salt linkages by increasing the ionic strength of the solvent. The mechanism by which these salts effect the solubility of microsomal membrane proteins may be elucidated in light of the recent findings of Hatefi and Hanstein (65). These authors investigated the effects of chaotropic ions, i.e., those ions which favor the transfer of apolar groups to water (e.g. SCN^-, NO_3^-, I^-, Br^-, and Cl^-) on the solubilization of particulate proteins. Using the solubilization of small organic molecules as a model they found the order of increasing salting-out effect of anions was essentially: $SCN^- < ClO_4^- < NO_3^- < I^- < Br^- < Cl^- < SO_4^-$, CH_3COO^-, F^-. It has also been found that this order also corresponds to the decreasing effectiveness with which these ions disrupt the structure of diverse macromolecules (59, 66, 67) and inhibited the activities of various enzymes utilizing both charged and

uncharged substrates (68, 69). In regard to these studies, the decrease in the effectiveness of solubilization of microsomal membrane proteins generally followed the order of ions given above. These observations were found to hold true for anions regardless of the identity of their corresponding cations. Hateifi and Hanstein (65) rationalized these data on the basis of the structure breaking effect that these anions have on water and because of their ability to make water more lipophilic. The structure breaking effects of these anions on water, which was found to increase in order of decreasing salting-out effects, were judged by: the high positive entropy content for the hydrated forms of these ions; the increased shielding of water protons (as determined from the chemical shifts observed by NMR); and the increased mobility of water molecules (determined from the self diffusion coefficients of pure water and the ionic solutions). These authors explain the increased lipophilicity of solutions of these anions as due to the fact that anions decrease the polarity of the surrounding water. This postulate was explainable by the rationalization of Greyson (70) that "the protons of the solvated water molecules polarizes the large halide ions and leads to a water-anion bond less polar than the OH---O of water itself, while the less polarizable cations tend to form hydrate bonds similar in polarity to that of water itself."

With regard to the explanations of the effects of chaotropic ion on the structure and lipophilicity of water mentioned above, some rationalization of the data obtained from these studies can be made. It appears that the effectiveness of the salts on the solubilization of microsomal membrane proteins parallels their ability to render water more lipophilic and in like manner, their ability to weaken the hydrophobic forces which have a major contribution in the maintenance of membrane integrity. The observation that lysis in 0.3 M glycerol and the control extraction of microsomes with Tris-HCl buffer solubilized to a lesser degree, essentially the same protein species as did extractions with chaotropic ions may also reflect a weakening of hydrophobic bonds by decreasing the polarity of the solvent. This is accomplished by decreasing the salt content with respect to physiological conditions and/or addition of a less polar solvent such as glycerol to water. The effects of polyvalent anions on microsomal membrane protein may be due to their ability to disrupt salt linkages, e.g. those postulated to be involved in maintaining structural integrity of ribosomes. Some support for the latter statement may be gained from the fact that the protein components of ribosomes are highly charged, predominately basic proteins (71).

EDTA-KCl extraction.--Combinations of reagents which would possibly disrupt the forces responsible for ribosomal

integrity, i.e., EDTA and KCl, were investigated for their ability to extract ribosomes. Of the combinations investigated (Table 8), washing microsomes with Tris-HCl buffer containing 0.01 M EDTA and 0.15 M KCl seemed most effective in extracting ribosomes. This conclusion was made on the basis of the quantitative studies performed on the extracted microsomal pellets, in which the percentage of RNA-ribose and non-lipoprotein solubilized were taken under consideration. These reagents probably exert their effects by removing Mg^{++}, which has been shown to cause an unfolding of the ribosomal 50_s and 30_s subunits (72), and disrupting the electrostatic interactions thought to be important in maintaining ribosomal structure.

The proteins extractable with Tris-HCl buffer, 0.01 M EDTA and 0.15 M KCl, are believed to be ribosomal. The observations leading to this conclusion is that this treatment extracts more RNA-ribose (Tables 4 and 8) and that the proteins extracted have been identified, electrophoretically, with proteins believed to be ribosomal (see Figure 1, tubes 2 and 6, also see the discussion given under Microsomal Extraction Procedures in the Discussion section of this thesis). One puzzling observation was made in that one of the protein bands extracted was shown to be a major protein constituent of a crude ribosomal preparation (Figure 6, tube 11) and of SER subfractions (Figure 1). It is believed that this particular band, seen to be specifically concentrated in the SER II subfraction shown in Figure 1,

represents a contaminating protein which strongly binds to SER and the crude ribosomal fraction. This explanation, however, is inconclusive in that the protein band in question also appears to be identical to the faster migrating of the two major protein species not extractable with KSCN (see Figures 3 and 4), which as discussed previously was shown to have a relatively high RNA-ribose content (Tables 5-7). It may be that KSCN solubilizes all of the microsomal membrane proteins with exception of some ribosomal proteins and SP. This, however, is mere speculation and the data observed may be explainable by some other means, but so far as these studies are concerned such data shall be termed inconclusive.

Combined treatments of microsomes.--Attempts were made to extract the faster migrating of the two major protein species seen in the electrophoresis profile of the proteins not extractable with 2.0 M KSCN (Figures 3 and 4). Table 9 shows the various treatments and the quantitation of total and lipid-phosphate and RNA-ribose from each extraction. The data from experiment number 1 shows that RNA-ribose is extractable with KSCN after exposure of microsomal protein to 1.5 M NaBr. This is contrary to other data obtained by treatment with KSCN alone (Tables 5-7) or after extraction with EDTA-KCl which all indicate that KSCN concentrates RNA-ribose. The effect observed by pretreatment of microsomes with 1.5 M NaBr followed by

2.0 M KSCN extraction could probably be explained by some structural alteration of microsomal protein caused by NaBr which would render RNA more susceptible to the action of KSCN. The fact that there was no differences in the electrophoresis profiles of microsomes extracted with 2.0 M KSCN alone or after the series of treatments listed in Table 9 (experiment 1) indicates that these combined treatments were useless with respect to protein solubilization.

Effects of Various Treatments and Extraction Procedures on the Enzymatic Activities of Microsomes

Effects of freezing, lysis, and EDTA-KCl washing.-- Before a discussion of the effects of various treatments on NADPH-cytochrome c reductase and aminopyrine demethylase activity some pertinent properties of these enzymes should be mentioned. NADPH-cytochrome c reductase is a flavin (FAD) containing enzyme (74) which is rather firmly attached to microsomal membranes (51); it has, thus far, been found extractable by treatments such as trypsin digestions of microsomes (75). Aminopyrine demethylase is one of several mixed-function oxidase activities and is dependent on enzymes involved in microsomal electron transport (31, 76); chief among these is cytochrome P-450. Alterations of demethylase activity could therefore be due to alterations in components of microsomal electron transport, a fact that should be kept in mind when interpreting these data.

Activation of NADPH-cytochrome c reductase and aminopyrine demethylase activities (Table 10) by glycerol and sucrose is not clearly understood. One could postulate, however, that the increase is due to alterations in microsomal membrane structure which could facilitate the availability of substrate to the enzymes involved. The observation, made earlier in these studies, that glycerol enhances solubilization of membrane proteins may lend support to this postulate. The increase seen in demethylase activity is apparently independent of reductase activity. This observation is made in view of the fact that freezing microsomes in Tris-HCl buffer (50% glycerol) does not effect the reductase activity whereas the demethylase activity is inhibited considerably. Loss of both reductase and demethylase activity by freezing microsomes in 0.25 M sucrose probably reflects protein denaturation. Evidence of this was mentioned previously (see Discussion, under Lysis of rat liver microsomes) in which a correlation was made between the method of storage and protein extractable by lysis. The apparent recovery of reductase and demethylase activities of the frozen microsomes may also be explainable in terms of this correlation. It was observed that essentially the same amount of protein was extractable by lysis of control and Tris-glycerol frozen microsomes whereas the protein extracted by lysis of sucrose-frozen microsomes was significantly less. This observation correlates, for example, with the reductase activities observed after lysis

of control and frozen microsomes, Table 10. The demethylase activities, however, do not show such correlations and probably involves other mechanism of activation not clearly understood.

The decrease in reductase and demethylase activities by washing microsomes with Tris-HCl buffer and Tris-HCl buffer, 0.15 M KCl may be rationalized in terms of an activation by glycerol rather than inhibition by Tris-HCl buffer or extraction of demethylase and reductase activities (Table 11). It must be mentioned that the control microsomes were those stored in Tris-HCl buffer (50% glycerol) and underwent no further treatment. The data in Table 10 conclusively shows that glycerol enhances reductase and demethylase activities. With this established, the effects of washing with Tris-HCl buffer or Tris-HCl buffer containing 0.15 M KCl, shown in Table 11 can be interpreted as a reversal of activation by removal of glycerol. In like manner washing with Tris-HCl buffer containing 0.01 M EDTA and 0.01 M EDTA + 0.15 M KCl would also be expected to reverse glycerol activation but the apparent increase in activity would be expected as a result of further solubilization of membrane proteins, e.g. ribosomal protein.

Salt extractions: Effects on enzymatic activities of microsomes.--It has been shown previously (Tables 5-7; Figures 3, 4, and 5) that the effects of extractions with salts containing monovalent anions and those containing

polyvalent anions were different with respect to the amounts and identities of the proteins solubilized. These salts also exhibited different effects with regard to reductase and demethylase activities, Tables 12 and 13. For example, K_2HPO_4 was found to activate aminopyrine demethylase activity (Table 13) but had no effect on reductase activity, Table 12. This suggests a possible activation of aminopyrine demethylase independent of NADPH-cytochrome c reductase, possibly through effects on microsomal electron transport components. Though it was shown (Figure 5) that polyvalent anion containing salts extract essentially the same protein species, it cannot be determined from these studies if the effects of K_2HPO_4 on reductase and demethylase activities are unique or whether these effects may be extrapolated to other polyvalent anion containing salts.

Salts containing monovalent anions, e.g. NaBr and NaCl, were shown to increase NADPH-cytochrome c reductase activity (Table 12) and to decrease aminopyrine demethylase activity at high substrate concentrations but not at low substrate concentrations (Table 13). These data indicate that aminopyrine demethylase activity is not limited by NADPH-cytochrome c reductase. Also the differences in the effects of salts on aminopyrine demethylase activity at different substrate levels (Table 13) give support to the postulate that more than one enzymatic component is responsible for aminopyrine demethylase activity. From this data it was shown that the enzymatic component(s) of aminopyrine

demethylase with the higher affinities for aminopyrine are activated to a greater extent by salts. Also there are indications that components of aminopyrine demethylase at low substrate concentrations are extractable with salts (Table 13, last column).

The exact mechanism by which these salts affect reductase and demethylase activities are not known; one can only postulate mechanisms, keeping in mind the effects these salts have on water. For example it has been shown that salts containing monovalent anions alter the structure of water and makes it more lipophilic (65). Also it has been shown that solutions of these salts can significantly alter or denature protein structure (65, 69). In regard to these findings one may suggest that the increased reductase activity shown by NaBr and NaCl reflect a favorable alteration in membrane or enzyme conformation which may facilitate substrate binding thereby enhancing enzymatic activity. The effects of salts on aminopyrine demethylase activities are difficult to explain. It appears that the component responsible for demethylation at higher substrate levels is inhibited, probably due to denaturation, by monovalent anions and that these anions activate the component responsible for demethylation at low substrate levels. The fact that K_2HPO_4, a salt not having the structure altering effects on water as does NaBr and NaCl, also activates this component may be

indicative of an ionic strength or cation effect. These mechanisms are highly speculative, however, and the effects of these salts on enzymatic activities warrant further investigation.

SUMMARY

In these studies microsomes were treated with a number of agents known to disrupt the forces responsible for attachment of proteins to the membrane and for the structural integrity of membranes. Among the methods investigated were organic solvent, salt, and EDTA-KCl extractions, and lysis in the presence of 2.76% glycerol. Extractions of microsomes with various organic solvents (e.g. dioxane-water, 1:4 and 4:1 v/v; pyridine; butanol; N,N'-dimethylformamide-water, 1:1 v/v; and 2-aminoethanol) showed correlations between the dielectric constants of the solvents used and the amount of protein solubilized. Also the presence of 0.1 M Mg^{++} in the organic solvent methods investigated resulted in an enhancement in protein solubilization by these extractions. The enhancement of solubilization by Mg^{++} is believed to result from denaturation of proteins by high Mg^{++} content, subsequently exposing more of the hydrophobic regions of proteins to the organic solvents.

Exposure of microsomal membranes to high salt concentrations at pH 7.5 was found to extract proteins in a manner such that their identity and relative amounts were

characteristic of the salts used. Salts containing monovalent anions, e.g. KSCN, NaBr, $NaNO_3$, and NaCl, were shown to solubilize microsomal membrane proteins in the same order of effectiveness with which they were shown to disrupt the structure and to increase the lipophilicity of water (65). Each of the monovalent anions investigated extracted essentially the same proteins, as determined by electrophoresis. Salts containing polyvalent anions, e.g. the sodium salts of citrate, phosphate, pyrophosphate, and carbonate, were shown to be less effective in extracting microsomal membrane proteins. These salts which do not exhibit the same structure breaking effects on water or change the lipophilicity of water as the salts containing monovalent cations were also shown to extract some proteins not extractable with monovalent anion containing salts. These proteins are believed to be ribosomal stemming from the observations made that: these proteins were observed to be characteristic of the RER fraction of microsomes and they were found to be extractable with Tris-HCl buffer containing 0.01 M EDTA and 0.15 M KCl, a mixture believed to be effective in ribosomal protein extraction. The combined observations, i.e., those concerning organic solvent extraction and monovalent anion extractions, mentioned above, suggests that the major forces involved in maintaining structural integrity of microsomal membranes are hydrophobic. Such forces probably express themselves both in the interactions between proteins and lipid

components forming the subunits (3, 4) of membranes and in the interactions between these various subunits. Ionic interactions combined with Mg^{++} complexing have been shown to be forces involved in the stabilization and/or attachment of ribosomes to microsomal membranes. This deduction was made in light of the facts EDTA in the presence of relatively high salt concentration or high salt concentrations (i.e., those containing polyvalent anions, which are believed only to effect buffer solutions by raising the ionic strength) alone.

Structural protein (SP), that apparently non-catalytic protein species believed to have a structural function (10, 43) in membranes, was investigated in these studies. Correlations were found in the basic structural organization of mitochondrial and microsomal membranes with respect to their membrane forming or basepiece and detachable portion. Green et al. (43) have evidence for there being two subclasses of non-catalytic proteins responsible for mitochondrial membrane structure, i.e., SP responsible for structural integrity of the detachable portion and core protein (CP) responsible for the structural integrity of the basepieces. Methods typically used to isolate SP (10, 13, 44, 45) have resulted in isolation of a protein from microsomes which would correspond to SP of mitochondria. Also a protein fraction has been purified here by 2.0 M KSCN extraction of microsomes which, because of its high lipid content, inducability in microsomes by a treatment

known to cause proliferation of microsomal membranes, and its predominance in microsomes, is believed to correspond to CP of mitochondria. This protein, however, after isolation appeared denatured and the true functional (i.e., structural and/or enzymatic) has yet to be demonstrated.

The harshness of the various treatments mentioned above on microsomal proteins was evaluated by their effects on NADPH-cytochrome c reductase and aminopyrine demethylase activities. The effects of storage of microsomes was determined and it was found that suspending microsomes in Tris-HCl buffer (50% glycerol) or 0.25 M sucrose increased both reductase and demethylase activities. Glycerol was found to protect reductase but not demethylase activity upon freezing; whereas, with sucrose freezing resulted in a considerable loss in both activities (from 3 to 20% of control). An activation of reductase activity by NaBr and NaCl, and of demethylase activity by K_2HPO_4, NaBr, and NaCl was also observed. The latter two salts, however, only activated the component of aminopyrine demethylase which had the higher substrate affinity; the component with the lower affinity was inhibited by these salts. Neither lysis of microsomes nor EDTA-KCl washings were found to extract reductase or demethylase activities whereas salt extractions, using NaBr and NaCl at concentrations from 0.5 to 1.0 M, did. Activation of reductase and demethylase activities is believed to result from conformational or

structural changes within the microsomal membranes which increase substrate availability to enzymatic sites.

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