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THE ISOLATION AND CHARACTERIZATION OF A MEMBRANE PROTEIN COMPONENT FROM RAT LIVER ENDOPLASMIC RETICULUM

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SUMMARY

A subfraction of membrane protein has been isolated from the microsomal fraction of rat liver. This material has blocked N-terminal amino groups and a distribution of C-terminal amino acids, indicating that the membrane protein preparation is composed of distinct chemical species. However, it has been demonstrated that the species present comprise a reversibly associating system. Furthermore, the characteristics of this system are such that the species involved are probably related to one another. Additional information shows that the protein is associated with nucleotide-like material in a ratio of 1 mole of nucleotide per 10000 g of protein. The preparation has also been characterized with respect to relative contents of cysteic acid, methionine sulfone, proline and glutamic acid. The ratios obtained from the experimental values may be used to identify the protein subfraction and distinguish the preparation from membrane protein obtained from other sources.

INTRODUCTION

Membrane proteins, believed to have a structural role, have been isolated from a variety of organelles, tissues and organisms (for recent review see ref. 1). One source of these proteins isolated in a preliminary investigation but subsequently neglected to a large degree was microsomal membranes[2]. The lack of further study of these proteins is rather surprising as endoplasmic reticulum, obtained as microsomal fraction, forms the most extensive membrane system in eucaryotic cells. In addition endoplasmic reticulum has been well characterized with regards to preparation, chemical and enzymatic compositions (see review of DALLNER AND ERNSTER[3]). An added advantage of working with endoplasmic reticulum is the ease with which it can be obtained free from other membrane systems particularly when liver is used as a source.

Microsomal membrane proteins, obtained both by extracting microsomes with alkali as well as by solubilization of membranes and subsequent removal of lipid, have been resolved into fifteen distinct bands by electrophoresis on polyacrylamide gels[4,5]. (The different gel systems as well as methods of isolation employed preclude direct comparisons of proteins from these two preparations.) Aggregation alone could

Abbreviation: dansyl, 1-dimethylaminonaphthalene-5-sulfonyl.

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not account for the observed multiple band patterns. Preparations of microsomal membrane protein by other workers also show great dispersity, as indicated by the formation of an asymmetrical peak in the analytical ultracentrifuge[6]. The reported sedimentation coefficient of the major components of this peak was 2.3 S.

Investigations into the binding properties of microsomal membrane protein have included studies of phospholipid binding *versus* pH[2], ATP binding[2,7], RNA binding[7,8] and specific protein binding[9]. The results of these binding studies strengthen the suggestion that the isolated protein retains some of its membrane protein characteristics by virtue of its ability to interact with components normally associated with membranes. In addition, there are distinct analogies between its binding properties and those of mitochondrial "structural protein". (For review of mitochondrial structural protein binding properties see ref. 1.)

Of additional interest in light of the recent discussion of miniproteins is the report of membrane protein isolated from sheep brain microsomes[10,11]. It fulfills published criteria of isolation, solubility and binding properties for proteins derived from other membrane structures. The protein preparation as isolated, had a molecular weight distribution of 90000–300000 g/mole based on Sephadex column chromatography. However, treatment with phospholipase A dissociated the protein so that two new classes of proteins having molecular weights of 40000 and 2000–12500 g/mole were observed. The dissociation was not attributable to any proteolytic activity of phospholipase A.

Information available to date thus defines the membrane proteins of endoplasmic reticulum only as a poorly characterized group of heterogeneous proteins. This communication presents information describing a further characterization of the membrane proteins for identification purposes and investigates chemical and physical relationships existing among the proteins present in a major subfraction of the microsomal membrane protein preparations.

METHODS AND MATERIALS

Trypsin (recrystallized twice), DFP-treated carboxypeptidase A, and DFP-treated carboxypeptidase B were obtained from Worthington Biochemical Corp. Ficoll was obtained from Pharmacia Fine Chemicals. The synthetic peptide, Val–Ala–Ala–Phe, was a gift from Dr. Edward Herbert. Bovine serum albumin was obtained from Pentex.

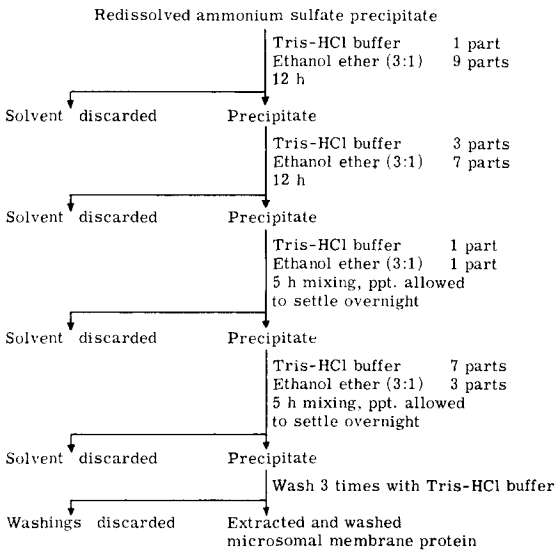
Isolation of microsomes

Male, Sprague–Dawley rats (125 g each) were killed by decapitation. The livers were removed and washed with 0.25 M sucrose at 0°. All subsequent operations were performed at 4°, except where noted. The livers were suspended in 0.25 M sucrose (1 ml per g wet wt.), disrupted in a Waring blendor for 15 sec, and then homogenized in a motor-driven glass–Teflon homogenizer. The liver suspension was diluted with 0.25 M sucrose (final volume: 2 ml suspension per initial g of liver), and centrifuged for 30 min at 12000 × *g*. The supernatant fraction was aspirated and centrifuged for 85 min at 30000 rev./min in a Spinco No. 30 rotor to pellet the microsomal fraction. The pellets from this centrifugation were resuspended with gentle homogenization in 0.25 M sucrose (final volume: 0.33 ml suspension per initial g of liver). An aliquot

was removed from the microsomal suspension in order to determine the yield of membrane protein.

Preparation of membrane protein

A solution of 8 % sodium deoxycholate, 4 % sodium cholate and 30 mM CsCl in 5 mM Tris–HCl buffer (pH 7.5) was added to the microsomal suspension in the ratio of 1:1 (v/v) to solubilize the microsomal membranes. The clarified microsomal suspension was centrifuged for 120 min at 50000 rev./min in a Spinco No. 50 rotor to pellet ribosomes. $NaHSO_3$ was added to the supernatant fraction (12.5 mg per 50 ml) and the solution was brought to 27 % satn. with a cold saturated solution of $(NH_4)_2SO_4$. The mixture was immediately centrifuged for 30 min at 17300 × *g*. The $(NH_4)_2SO_4$ precipitate was redissolved in 1/6 the volume of the original $(NH_4)_2SO_4$–membrane mixture using Tris–HCl buffer. The residual pellet in the centrifuge tubes was solubilized in Tris–HCl buffer by washing with 1/12 the volume of mixture and the washings combined with the redissolved precipitate. The solubilized precipitate was extracted at room temperature using 25 vol. of each of four solvents in series, for every volume of original microsomal suspension according to Scheme 1. A portion



Scheme 1. Scheme for extraction of lipids from microsomal membrane protein. See text for additional details.

of the extracted and washed material was solubilized by stirring overnight at room temperature with 5 vol. (relative to the original volume of microsomal suspension) of a solution of 1 % sodium dodecyl sulfate in Tris–HCl buffer. The suspension was then centrifuged for 20 min at 17300 × *g* and the precipitated residue discarded. A sample was taken to determine the yield of solubilized protein from the total microsomal suspension. The remainder of the material was stored at 4°.

A portion of microsomal suspension was brought to 1 % sodium dodecyl sulfate by the addition of a 10 % aqueous solution of sodium dodecyl sulfate. The yield was determined with biuret reagent[12] using bovine serum albumin as a standard.

Physical studies

Dry weight of a sample that had been exhaustively dialysed against 1 % sodium dodecyl sulfate in Tris–HCl buffer, was determined on a Cahn RG vacuum electrobalance. The biuret-positive material in an equivalent sample was also determined.

An investigation of the absorption spectrum for characterizing protein and associated nucleic acid was performed by treating part of the protein solution with $HClO_4$ and comparing it spectrophotometrically with the untreated preparation. The following mixtures were prepared: (1) protein–water–70 % $HClO_4$ (1:1:1, by vol.); (2) protein–water (1:2, by vol.); (3) 1 % sodium dodecyl sulfate, Tris–HCl buffer–water–70 % $HClO_4$ (1:1:1, by vol.); (4) 1 % sodium dodecyl sulfate, Tris–HCl buffer–water (1:2, by vol.). The mixtures were centrifuged at 12000 × *g* for 30 min and any precipitated protein discarded. The absorption spectra of the supernatant fractions were recorded from 230 to 310 nm on a Cary-11 recording spectrophotometer using the following paired combinations (sample *versus* blank): 4 *vs.* 4, 2 *vs.* 4, 3 *vs.* 4, 1 *vs.* 4, 2 *vs.* 1. The molar extinction coefficient of any 260-nm absorbing material was assumed to be 10000 (average value for a nucleotide at acid pH).

Additional physical information was obtained by sedimentation velocity and sedimentation equilibrium experiments. Material was prepared for the sedimentation velocity experiment by cooling it to 0° and removing precipitated sodium dodecyl sulfate by centrifuging the suspension for 20 min at 17000 × *g*. 85 % of the protein was recovered in the supernatant fraction. This fraction (protein concentration: 2.4 mg/ml) was centrifuged at 60000 rev./min (20°) in a Spinco Model E ultracentrifuge. The protein solution used for sedimentation equilibrium experiments was prepared by precipitation of excess sodium dodecyl sulfate as above and dialysis against Tris–HCl buffer (0.05 M), 0.01 % sodium dodecyl sulfate (pH 7.5) for 48 h. Three concentrations of protein, 0.007, 0.0098 and 0.014 %, were prepared by dilution of this solution with dialysate. Each dilution was centrifuged at 30000 rev./min (23.5°) for a minimum of 24 h in a double-sector cell in a Spinco Model E ultracentrifuge. The meniscus depletion technique described by YPHANTIS[13] was used for molecular weight determination. The interference patterns on glass plates were measured with a Nikon microcomparator. The molecular weights were calculated using a computer program devised by SMALL AND RESNIK[14] and modified by S. LOWE AND M. KELLY (personal communication). The partial specific volume of the protein was calculated to be 0.743 ml/g using the method of COHN AND EDSALL[15] from amino acid analysis data of whole microsomal membrane protein (D. KAPLAN, unpublished data), and the density of the solution was estimated to be 1.006 g/ml. No correction was made for bound sodium dodecyl sulfate.

Chemical studies

Phosphorus content was determined by the method of CHEN *et al.*[16], using the ashing procedure of AMES AND DUBIN[17]. Sugar content was determined colorimetrically using glucose as the standard[18]. In addition, the relative contents of cysteic acid, methionine sulfone, proline and glutamic acid in the performic acid-oxidized preparation were determined using a modified oxidation procedure of BAILEY[19]. Lyophilized protein was dissolved (5 mg/ml) in precooled (4°, 30 min) performic acid. The reaction

mixture was incubated at room temperature for 30 min, diluted 10-fold with water and lyophilized. After hydrolysis in 6 M HCl at 110° *in vacuo* for 24 h, the relative content of the four amino acids in the preparation was determined (in triplicate) using a Spinco 120B amino acid analyzer.

1-Dimethylaminonaphthalene-5-sulfonyl (dansyl) chloride was used to identify and determine the number of N-terminal amino acids obtainable from performic acid-oxidized and unoxidized protein[20–22]. After exhaustive dialysis to remove Tris (and performic acid oxidation where appropriate), the lyophilized preparation was resuspended in a 1 % aqueous solution of sodium dodecyl sulfate. Three types of samples were prepared; one containing 1.5 mg of membrane protein, a second with 1.5 mg of membrane protein *plus* 40 nmoles of trypsin, and a third containing only 40 nmoles of trypsin. All samples were lyophilized and reconstituted with 0.1 ml of 0.1 M $NaHCO_3$ (pH 8.5) to which 0.1 ml of dansyl chloride, 20 mg/ml in 2-chloroethanol, was added. The tubes were capped and incubated at room temperature overnight in the dark. Samples were diluted 10-fold with water and exhaustively dialysed against water. The dialysed samples were transferred to digestion tubes, lyophilized and hydrolysed in 6 M HCl at 110° *in vacuo* for 48 h. After hydrolysis, the samples were dried and redissolved in acetone–0.1 M acetic acid (3:2, by vol.). Portions containing 0.3 mg of membrane protein and 10 nmoles of trypsin were applied to silica-gel sheets (Eastman-Kodak Type K301R), along with standards. One-dimensional chromatography was performed in two solvent systems: (1) benzene–pyridine–propionic acid (80:20:2, by vol.); and (2) chloroform–ethanol–propionic acid (152:16:12, by vol.). The solvents were removed with a stream of air and the chromatograms were examined using an ultraviolet lamp.

Carboxypeptidase A was solubilized using the method of AMBLER[23, 24] and used in a ratio of enzyme to protein of 1:50 (w/w). Carboxypeptidase B was used in a ratio of 13.5 units per mg of protein.

The membrane protein solution was prepared by removal of excess sodium dodecyl sulfate as previously described. The supernatant fraction was concentrated by immersion in a 30 % solution of Ficoll in Tris–HCl buffer. It was then dialysed for 48 h against 0.2 M $NaHCO_3$, 0.01 % sodium dodecyl sulfate (pH 8.5). The final protein concentration was 13.0 mg/ml.

Carboxypeptidase A and B activities were verified by incubating a synthetic peptide, Val–Ala–Ala–Phe, with these enzymes both in the presence and absence of the membrane protein.

It was found in preliminary experiments that amino acids released from membrane protein by digestion with the carboxypeptidases were being adsorbed to the protein. To determine the amount of each amino acid adsorbed, a mixture of amino acids (Calibration Mixture, Beckman Instr., Spinco Div.) was added to the protein in three different proportions (63.7, 43.5 and 29.1 nmoles of each amino acid per mg protein dry weight). Separate portions of membrane protein along with the appropriate blanks were incubated with each enzyme under conditions identical to those for the synthetic peptide and amino acid controls. All solutions were incubated at 37° for 24 h. They were then acidified to pH 1 with 6 M HCl and the precipitated protein removed by centrifugation. The supernatant fractions were analysed for free amino acids on a Spinco 120B amino acid analyzer.

RESULTS

Protein determinations for yield and dry weight

The yield of sodium dodecyl sulfate-soluble protein from the total microsomal fraction was 18.4 %. The solubilized protein represented approx. 30 % of the residual material after lipid extraction of the $(NH_4)_2SO_4$ precipitate pellet. Further washing of the sodium dodecyl sulfate-insoluble residue with 1 % sodium dodecyl sulfate did not solubilize a significant amount of protein.

The ratio of protein as determined by biuret reagent to that determined by dry weight was 1.3. Protein was routinely determined by biuret reagent and converted to a dry weight value using this value.

Sedimentation velocity and sedimentation equilibrium determinations

The sodium dodecyl sulfate-soluble material sedimented as an asymmetrical peak with a sedimentation coefficient of 2.7 S (calculated for the maximum ordinate of the peak). This value was not corrected for sodium dodecyl sulfate contribution to solvent density or viscosity.

TABLE I

MOLECULAR WEIGHTS FROM SEDIMENTATION EQUILIBRIUM DATA

Molecular weights in g/mole.

Concn. of protein (%)	M_n		M_w		M_z	
	Meniscus	*Bottom*	*Meniscus*	*Bottom*	*Meniscus*	*Bottom*
0.007	26 900	40 800	33 400	46 400	42 300	52 100
0.0098	31 000	43 500	34 400	48 100	41 000	54 300
0.014	29 200	43 100	40 200	48 000	40 600	53 100

A summary of the results of the sedimentation equilibrium experiments is presented in Table I. A graph of the apparent weight-average molecular weight at each point in the cell *versus* the concentration at each point in the cell (determined by the deflection of interference fringes), was constructed for each of the three concentrations used (Fig. 1). The majority of points for all three concentrations lie near the same curve.

C-terminal analysis

When carboxypeptidase A or B were added to a solution of the synthetic peptide 100 % of the expected phenylalanine was released from the C-terminal position indicating that the reaction conditions were suitable for digestion of the peptide.

Control experiments in which amino acid was added to the protein solution indicated that basic and nonpolar amino acids were adsorbed by the protein and acidic amino acids were released from the protein (Table II). The results of the carboxypeptidase digestion of the protein preparation are presented in Table III. These values have been corrected for adsorption (or release) of amino acids by the

precipitated protein, and are presented as moles of amino acid per mole of protein (using an assumed molecular weight of 20000).

N-terminal analysis

There were no fluorescent spots attributable to dansyl derivatives observed for either the oxidized or the unoxidized protein preparations other than those derived from trypsin, ε-aminolysine, and dansyl amine. Under the conditions described it would have been possible to see as little as 1 % of the expected yield of a protein component with a molecular weight of 20000.

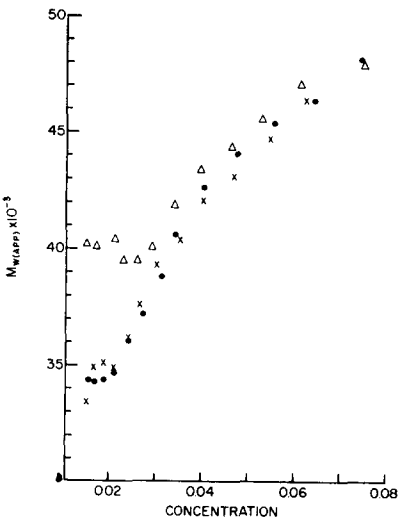


Fig. 1. Point values of apparent weight-average molecular weight. ×, 0.007 % protein; ●, 0.0098 % protein; △, 0.014 % protein.

TABLE II

PERCENT AMINO ACID ADSORBED BY PROTEIN

Amino acid	*Percent adsorbed by protein*			*Percent retained average*
	63.7 nmoles/mg	*43.5 nmoles/mg*	*29.1 nmoles/mg*	
Lys	13.3	24.8	22.7	23.8
His	23.9	28.1	25.8	25.8
Arg	47.1	51.9	56.8	51.9
Asp	— 1.8	— 6.4	—	— 4.1
Thr	— 7.1	—35.9	—	— 7.1
Ser	—12.4	—14.7	—	—13.6
Glu	— 0.6	— 2.5	4.5	0.5
Gly	— 0.6	— 2.5	0.3	— 2.0
Ala	3.9	— 1.3	5.5	2.7
Val	15.5	12.9	14.7	14.7
Met	20.7	18.9	27.2	22.3
Ile	34.2	34.5	48.8	35.8
Leu	39.1	39.3	47.8	40.5
Tyr	25.4	34.7	28.5	29.5
Phe	51.8	52.7	53.0	52.5

TABLE III

CORRECTED VALUES OF AMINO ACIDS RELEASED BY CARBOXYPEPTIDASE A AND B

Mole amino acid per mole protein: assumed molecular weight 20000 g/mole.

Amino acid	*Released by carboxypeptidase A*	*Released by carboxypeptidase B*
Lys	0.10	0.09
Arg	0.00	0.10
His	0.01	0.00
Asp	0.04	0.08
Thr	0.02	0.00
Ser	0.07	0.00
Glu	0.00	0.01
Gly	0.04	0.02
Ala	0.10	0.00
Val	0.09	0.04
Met	0.02	0.00
Ile	0.04	0.00
Leu	0.18	0.05
Tyr	0.10	0.00
Phe	0.16	0.09

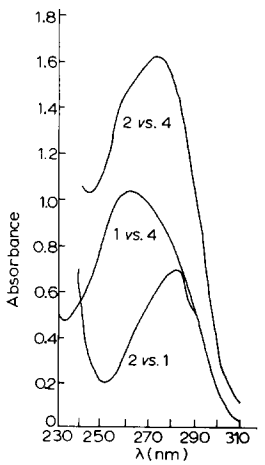


Fig. 2. Spectra of $HClO_4$-treated protein solution. (1) protein–water–70% $HClO_4$ (1:1:1, by vol.); (2) protein–water (1:2, by vol.); (4) 1% sodium dodecyl sulfate, Tris–HCl buffer–water (1:2, by vol.). See text for additional experimental details.

Phosphorus, sugar and ultraviolet spectra determinations

The phosphorus content was 0.13 μmole/mg protein, and sugar was 0.15 μmole/mg protein. Using the assumed molar extinction of 10000, a value of 0.12 μmole of nucleotide per mg protein was obtained for the $HClO_4$-treated sample (Fig. 2). Furthermore, when the untreated protein (2) was scanned using the $HClO_4$-treated sample (1) as the blank, a normal protein absorption spectrum with a prominent shoulder at 290 nm was observed.

TABLE IV

AMINO ACID ANALYSIS AFTER PERFORMIC ACID OXIDATION

Amino acid molar ratios	*Microsomal protein preparation*	*Mitochondrial structural protein*	
		Neurospora (ref. 27)	*Beef heart (ref. 25)*
Cys/Met	1.87	1.33	2.58
Cys/Glu	0.39	0.18	0.85
Cys/Pro	0.63	0.40	2.34
Met/Glu	0.21	0.14	0.33
Met/Pro	0.34	0.30	0.91
Pro/Glu	0.62	0.45	0.36

Performic acid oxidation

The results of the amino acid analysis of the performic acid-oxidized preparation are presented in Table IV as ratios of the four amino acids to one another. The ratios of these same amino acids as they occur in both beef heart mitochondrial structural protein, are presented in the same table.

DISCUSSION

While it is true that the microsomal fraction from most tissues is a mixture of intracellular membranes, that fraction isolated from liver is composed of smooth and rough endoplasmic reticulum without significant contributions from other membranes[3]. It was for this specific reason that we isolated the microsomal fraction we used in these studies from liver and did not attempt to further characterize it with respect to possible contamination by other intracellular membrane systems.

The sodium dodecyl sulfate-soluble protein represents 18.4 % of the total microsomal fraction and approx. 30 % of the total material present after lipid extraction. This figure is compatible with previously reported yields for membrane fractions from other sources[1].

In addition the sodium dodecyl sulfate-soluble material appears to be essentially free of lipid as indicated by the good correspondence between the phosphorus, sugar and spectral values. It is clear from these results that the phosphorus and sugar must be part of a nucleotide(s) and not derived from lipids or glycolipids.

Furthermore the nucleotide provides a minimal binding molecular weight of 10000 g/mole for the protein, if it is assumed that one nucleotide is bound per mole of protein. The minimal combining weight reported for mitochondrial membrane protein is 22500 g/mole (ref. 1). This suggests that the protein may in fact be associated with 2 moles of nucleotide per mole of protein of 20000 g/mole.

Previous reports of amino acid composition of membrane proteins indicated that many of these proteins were chemically similar to one another. In order to define chemical characteristics useful for identification of the microsomal membrane protein and for distinguishing this protein from other membrane proteins, the relative contents of cysteic acid, methionine sulfone, proline and glutamic acid were determined. These amino acids were specifically chosen because all are resistant to destruction during acid hydrolysis of the protein. In addition, three of them are normally present in

proteins in small quantities so that variations relative to one another are readily seen. The results indicated that the microsomal protein is chemically distinct from beef heart and Neurospora mitochondrial structural protein. It is therefore appropriate that these values be used to identify microsomal membrane protein and to distinguish it from other systems.

Originally it was claimed that the mitochondrial membrane proteins were homogeneous[25]. Subsequently it was demonstrated by acrylamide-gel electrophoresis that a pattern more characteristic of an unrelated, heterogeneous group of proteins than of a homogeneous aggregating system could be produced. Though similar patterns can be produced for the microsomal protein[5], they have not been presented here because preliminary experiments indicated that this apparent heterogeneity may not be a true reflection of the composition of this system.

To investigate this possibility, analysis of the N-terminal and C-terminal amino acids was undertaken. The results of the N-terminal analysis clearly indicate that in spite of adequate reaction conditions, free N-terminal amino groups were unavailable for reaction with the reagent. It may therefore be concluded that within the limits of detection, there were no free N-terminal amino groups in the membrane protein preparation.

The results of the C-terminal analysis are not as free of ambiguity. There are two possible explanations for the observed results. The first is that there is significant self association which prevents effective digestion of the C-terminal portion of all the chains. That is, the low yields, in terms of mole percent, primarily reflect incomplete digestion. The second interpretation is that the system is chemically disperse, so that the amino acids released actually reflect the C-terminal portion of different chains. This latter interpretation is strengthened by the rather high total yield of amino acids of 107 % (in terms of mole percent) obtained when the protein is treated with carboxypeptidase A.

Though chemical dispersity is suggested by the results of the carboxypeptidase A digestion, it is unequivocally demonstrated when these results are compared to those derived from digestion of the protein with carboxypeptidase B. No arginine is released by treatment of the protein with carboxypeptidase A, but an amount equivalent to lysine is released when the protein is digested with carboxypeptidase B. In both digestions lysine is released in approximately the same amounts, even though most of the other amino acids are released in substantially lower quantities by carboxypeptidase B than they are by carboxypeptidase A. A necessary condition is that lysine and arginine are either on the same chain or on different ones. If the latter is true the system is disperse. If lysine and arginine are part of the same chain, there must also be at least one other chain in order to account for all the amino acids released by carboxypeptidase A. (Note: The high yield of lysine, an amino acid normally released slowly by carboxypeptidase A may reflect a change in the apparent susceptibility of this residue to digestion because of association with sodium dodecyl sulfate.)

At this point it may be proposed that the membrane protein preparation is composed of proteins with covered N-terminal amino acids and probably with a distribution of C-terminal amino acids. It is appropriate then to ask, what is the physical relationship if any, among these different chemical moieties? To investigate this question we have employed the techniques of analytical ultracentrifugation.

The results of the sedimentation velocity experiment simply indicate a physi-

cally disperse system, but nothing about the relationship among the solution components. However, the results of the sedimentation equilibrium experiments are very revealing in this respect.

The curves shown in Fig. 2 exhibit characteristics described by VAN HOLDE AND ROSSETTI[26] as being uniquely attributable to reversibly associating systems. Such systems are by definition ones in which all species are in equilibrium with one another. There are two models based on reversibly associated systems fitting the observed distribution of molecular weights seen for the microsomal protein. The first and simplest is a system composed of identical or related protein species forming an equilibrating system, *i.e.* of monomer–dimer components. This explanation is compatible with the values for the lowest molecular weight (from M_n) and the highest molecular weight (from M_z) species observed. The second explanation involves a system of rapid equilibration with the primary species. Certain qualifications can be made about the nature of these unrelated species and about the system as a whole. (1) All species take part in the formation of the complex as it is an equilibrating system. (2) The smallest observed species in the system is about 26000 in molecular weight. (3) The complex must be larger than any of the primary species and therefore has a molecular weight greater than or equal to the largest observed species, *i.e.* that of about 52000 g/mole in molecular weight. (4) There is no primary species present which is much larger than about 26000 g/mole in molecular weight because the associated complex is only about 52000 g/mole or slightly larger in molecular weight. Therefore, the unrelated primary species are all about the same size. Furthermore, as the complex is formed and yet remains in an easily shifted equilibrium state, it may be concluded that the forces involved in the interactions among the primary species forming the complex are not very strong. In addition, it is noted that there appear to be no secondary complexes formed because the species of 52000 g/mole in molecular weight is the largest that is observed. This latter fact indicates that the forces promoting the interactions must be rather specific. The precedent for this claim of interaction specificity has been shown to be capable of specific interactions between itself and other proteins such as diphtheria toxin, myoglobin and cytochrome *c* (ref. 9). However, it does not interact with serum albumin, γ-globulin, hemoglobin or diphtheria toxoid. This lack of interaction with the last protein is of special significance because it shows the specificity of the interaction between diphtheria toxin and membrane protein.

In addition it has been reported that membrane protein has been resolved into fifteen discrete bands[4,5], some of which must be distinct physical species as aggregation alone could not account for the observed distribution of the multiple band in the pattern.

When the specificity of interaction and the presence of distinct physical species in the membrane protein preparation are considered, it is clear that the interactions among these species (seen by sedimentation equilibrium) must be taking place between a small number of similar sites on the proteins. If this were not true and there were multiple dissimilar sites then it would be very likely that much higher molecular weight components would be observed due to simultaneous interactions among several species. Instead only interactions between two (or three) protein molecules is observed, showing a restricted capacity for interactions among primary species and a marked absence of interactions between dimer (or trimer) complexes.

Therefore, from the two preceding arguments it can be concluded that, regard-

less of which explanation is chosen to account for the observation of the microsomal protein as a reversibly associating system, the species involved are similar to one another. It is only the degree of similarity which remains in doubt and this cannot be resolved by any further analysis of the data obtained from the sedimentation equilibrium experiments.

CONCLUSIONS

What little information has been available for characterizing the physical composition of microsomal membrane protein has indicated a heterogeneous system[4,5]. The data presented here serves to extend and in part quantitate these initial observations.

The preparation may be characterized as one free of lipid but tightly associated with nucleotide in an amount of 1 mole of nucleotide per 10000 g of protein. In addition it is chemically distinct from either beef heart or *Neurospora crassa* mitochondrial structural protein.

It is probably composed of chemically distinct proteins as evidenced by the C-terminal analysis, that have in common unreactive or blocked N-terminal amino groups. Furthermore, these species of protein interact reversibly, indicating that they are physically related to one another.

If in spite of our choice of liver as the source of the microsomal fraction we have been dealing with a fraction contaminated by other intracellular membranes it is significant that the proteins from these other membranes show sufficient similarity to the microsomal protein to interact in the fashion described.

It may be suggested in conclusion that these proteins, though dissimilar in some chemical and physical characteristics, represent a group of closely related proteins making up a discrete isolable subfraction of microsomal membrane proteins.

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