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SOLUBILIZATION, DISAGGREGATION AND CHROMATOGRAPHY OF ERYTHROCYTE MEMBRANES*

RICHARD B. TRIPLETT, JOAN SUMMERS, D. ELLEN ELLIS AND KERMIT L. CARRAWAY

Department of Biochemistry, Oklahoma State University, Stillwater, Okla. (U.S.A.)

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SUMMARY

Three different procedures have been used for solubilization, disaggregation and chromatography of erythrocyte membranes. Separation of lipid and protein of the membrane could be demonstrated in each system. No evidence could be found by any of the procedures for the presence of a significant amount of low molecular weight protein. A fourth system, using guanidine hydrochloride and cetyltrimethylammonium bromide, effected solubilization of the membrane but failed to disaggregate membrane proteins either from lipid or from each other.

INTRODUCTION

The morphology of biological membranes is based on the association of lipid and protein into supramolecular structures. The understanding of these structures requires a knowledge of the number and distribution of protein or polypeptide chains and of their abilities to combine with lipids. Electrophoretic methods, especially polyacrylamide gel electrophoresis, offer superior techniques for analyzing polypeptide distribution[1-7]. However, they are usually ill-suited to preparative separations, and, in some cases, can produce technical artifacts which lead to misinterpretation of results[8]. A combination of column chromatography and electrophoresis often is preferable for membrane analysis. In the present work several column chromatographic systems for separating components of erythrocyte membranes are described. The columns for separation of low molecular weight materials were chosen for initial investigations because they permit an analysis of the disaggregation of lipid and protein. This separation is critical to the understanding of the solubilizing power of the membrane solvent, and it is also often a prerequisite to complete protein fractionation, since lipid and protein peaks may overlap on columns that separate larger molecules.

MATERIALS AND METHODS

Materials were obtained from the following sources: sodium dodecyl sulfate, guanidine hydrochloride, cetyltrimethylammonium bromide and cytochrome *c* from

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Sigma; [^{3}H]acetic anhydride from Amersham/Searle; [^{14}C]cholesterol from New England Nuclear; Bio-Gel P-100 and Bio-Gel A-15M from Bio-Rad; Sephadex LH 20 and Blue Dextran 2000 from Pharmacia. Sodium dodecyl sulfate, cetyltrimethylammonium bromide and guanidine hydrochloride were recrystallized from ethanol before use.

Amino acid analyses were performed on a Beckman Model 120 C Amino Acid Analyzer after hydrolysis of the protein in 6 M HCl at 110 °C for 22 h[9]. Radioactivity was determined by scintillation counting using Bray's solution[10]. The protein profiles from the columns were determined from the absorbance at 280 nm or by ninhydrin analysis[11]. The carbohydrate profiles were determined by the anthrone procedure[12].

Blood was obtained from the Dallas Community Blood Bank, Dallas, Texas, in acid–citrate–dextrose solution. Both fresh (less than 1 week old) and outdated (3–5 weeks old) blood samples were used. Erythrocyte membranes were prepared from fresh human blood according to the procedure of Dodge *et al.*[13]. After the membranes were washed free of hemoglobin, they were packed at 40000 × *g* for 1 h.

Solubilization and chromatography in sodium dodecyl sulfate

1 vol. of packed ghosts was added to 1 vol. of 6 % sodium dodecyl sulfate and 0.02 % sodium azide in 0.1 M phosphate, pH 7.8. The sample was made 1 % in mercaptoethanol and allowed to stand 18–20 h at room temperature under a N_2 atmosphere. [^{14}C]Cholesterol (2–5 μl) was added and the sample was chromatographed in 1 % sodium dodecyl sulfate and 0.02 % sodium azide in either 0.05 M Tris (pH 8.0) or 0.05 M sodium phosphate (pH 7.0) on Bio-Gel P-100.

[^{3}H]Acetic anhydride labeling of ghosts

5 ml of packed ghosts in isotonic phosphate, pH 7.4, were allowed to react with 2 mCi of [^{3}H]acetic anhydride for 30 min at room temperature[14]. The ghosts were dialyzed overnight against 0.02 % sodium azide at 4 °C to remove unreacted [^{3}H]-acetic anhydride. The labeled ghosts were collected after centrifugation at 40000 × *g* for 1 h. The sample was prepared for chromatography as described above or stored at —20 °C.

Solubilization and chromatography in phenol–acetic acid–water or butanol–acetic acid–water

Solubilization of ghosts was achieved by the addition of 1 vol. of phenol or butanol and 1 vol. of acetic acid to 1 vol. of packed ghosts. Chromatography was carried out on a 3.5 cm × 45 cm Bio-Gel P-100 column in phenol–acetic acid–water, 1:1:1 (w/v/v) or on a 2.5 cm × 35 cm Sephadex LH 20 column in butanol–acetic acid–water (1:1:1, v/v/v).

Solubilization and chromatography in cetyltrimethylammonium bromide–guanidine hydrochloride

To 0.4 ml of [^{3}H]acetic anhydride-labeled ghosts was added 0.1 ml of 10 % cetyltrimethylammonium bromide. The sample was mixed vigorously, 2.5 ml of 6 M guanidine hydrochloride were added and the mixing was repeated until complete clarification of the solution was obtained. After adjustment of the pH to 8.5, the mixture was made 1 % in mercaptoethanol and allowed to stand overnight at room tempera-

ture under a N_2 atmosphere. The mixture was then taken to pH 6.5 and a 0.5-ml aliquot was chromatographed on a 1.5 cm × 90 cm P-100 column or a 1.5 cm × 75 cm Bio-Gel A-15M column. The columns were eluted with 5.5 M guanidine hydrochloride which contained 0.1 % mercaptoethanol.

Rechromatography of fraction from guanidine hydrochloride column in sodium dodecyl sulfate

The total fraction obtained from chromatography in guanidine hydrochloride was dialyzed against 0.02 % sodium azide at room temperature for 2 days with four changes of dialyzing solution. It was dialyzed 1 day against 1 % sodium dodecyl sulfate at room temperature, lyophilized and redissolved in 0.5 ml water. To this was added 0.5 ml of 6 % sodium dodecyl sulfate and 0.02 % sodium azide in 0.1 M phosphate, pH 7.8, and the mixture was incubated with 1 % mercaptoethanol overnight at room temperature under a N_2 atmosphere. The sample was chromatographed on a Bio-Gel P-100 column with 1 % sodium dodecyl sulfate and 0.02 % sodium azide in 0.05 M Tris, pH 8.0.

Preparation of ^{32}P and ^{3}H doubly labeled erythrocyte membranes

Washed red cells were labeled* and washed by the procedure of Philippot[15], using [^{32}P]phosphate (New England Nuclear). Ghosts were prepared from these cells as described and labeled a second time with [^{3}H]acetic anhydride. The double-labeled membranes were washed and then solubilized in either sodium dodecyl sulfate or guanidine–cetyltrimethylammonium bromide for chromatography.

RESULTS

Chromatography in sodium dodecyl sulfate solutions

Erythrocyte membranes can be separated into two different fractions by chromatography on Bio-Gel P-100 in 1 % sodium dodecyl sulfate. Fig. 1 shows the profiles for protein, carbohydrate and externally added [^{14}C]cholesterol. The distribution of these components suggests that the fractions represent the protein and lipid of the membrane. This was also shown by sodium dodecyl sulfate acrylamide electrophoresis of the two fractions. The first showed the same pattern of proteins as the whole erythrocyte membrane[2], while the second showed only a band (detected by the periodate–Schiff method[16]) corresponding to the position at which lipids are detected on sodium dodecyl sulfate acrylamide gels[2,3] (Fig. 2). The column chromatographic elution positions of the two fractions can also be monitored by radioactivity assay if the membranes are prelabeled with radioactive acetic anhydride[14]. This method offers a very quick and sensitive method of assay which has been used in additional column chromatographic studies. Fig. 3 shows the elution profile of a sample of labeled human erythrocyte membranes. Both protein and lipid fractions can be labeled, since both contain free amino groups. The absorbance profile indicates that there is little protein material present except in the protein peak which occurs at the void volume. In order to determine quantitatively the amount of protein eluted in the two peaks, all fractions containing radioactivity for each peak were

* Labeling experiments with ^{32}P were performed by Dr B. C. Shin.

combined. Samples of these were hydrolyzed and subjected to amino acid analysis. Table I shows the amino acid analysis of the separated fractions for a typical chromatographic separation. Direct hydrolysis in the presence of sodium dodecyl sulfate was performed to prevent losses of peptides during detergent removal, but this procedure does not permit analysis of amino acids which are susceptible to oxidation.

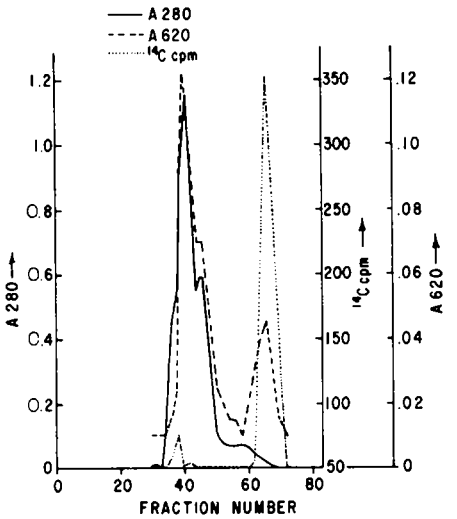


Fig. 1. Chromatographic profile of bovine erythrocyte membranes eluted from P-100 column (2.5 cm × 90 cm) with 1% sodium dodecyl sulfate. Void volume, Fraction 37. Protein (———), carbohydrate (-------) and [^{14}C]cholesterol (· · · · · ·).

Otherwise the analysis compares favorably to those of untreated membranes. Based on the amino acid analysis results from several chromatographic experiments on human erythrocyte membranes, the amount of protein eluted after the void volume peak was shown to be less than 5% of the total protein. Carbohydrate in the protein peak represented 45–55% of the total. Failure to observe ethanolamine in the protein peak suggests that the protein contains little complexed lipid and that lipid is disaggregated from protein. Since this observation represents a rather insensitive determination of lipid content, additional experiments were performed with membranes double labeled with [^{32}P]phosphate and [^{3}H]acetic anhydride. By this method 10–20% of the total phosphorus was eluted with the protein peak. Since this amount is apparently too great to be labeled phosphoprotein[17], these experiments indicate that phospholipid is not completely separated from the protein in this system. More complete disaggregation and separation were achieved by sodium dodecyl sulfate acrylamide electrophoresis (B. C. Shin, unpublished observations).

Comparison of the position of the lipid peak with those of cytochrome *c* and sucrose (Fig. 3) indicates that the lipid exists as an aggregate. This is also shown by the coincidence of the positions of the cholesterol and phospholipid on both the elution profile by sodium dodecyl sulfate chromatography and the migration profile by sodium dodecyl sulfate electrophoresis[14]. Laico *et al.*[18] have suggested that the protein of the erythrocyte membrane is an aggregate of lower molecular weight species that are partially disaggregated in sodium dodecyl sulfate solution in an equilibrium

system. However, repetitive chromatography of the protein peak from the experiment of Fig. 3 showed that no further disaggregation was achieved, as shown by the single radioactive peak eluted at the void volume of the column.

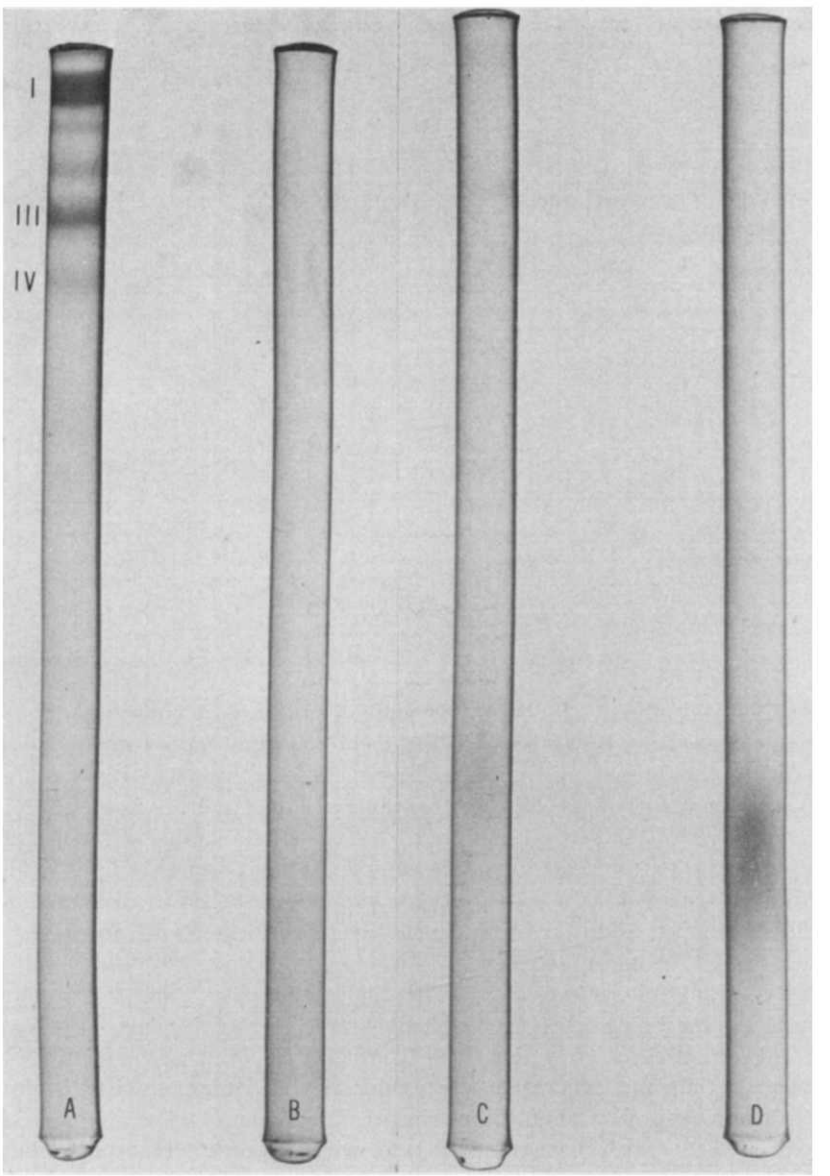


Fig. 2. Acrylamide electrophoresis of fractions from P-100 column. Column fractions were applied directly to gels without concentration. Gels were run, stained and destained as previously described[14]. Gel A, protein fraction stained with Coomassie Blue. Bands are numbered as described in previous work[32]. Minor components with lower molecular weights (V–VIII) were too faint to be recorded in our photographic system. Gel B, lipid fraction stained with Coomassie Blue. Gel C, protein fraction stained with periodate–Schiff method; Gel D, lipid fraction stained with periodate–Schiff procedure.

TABLE I

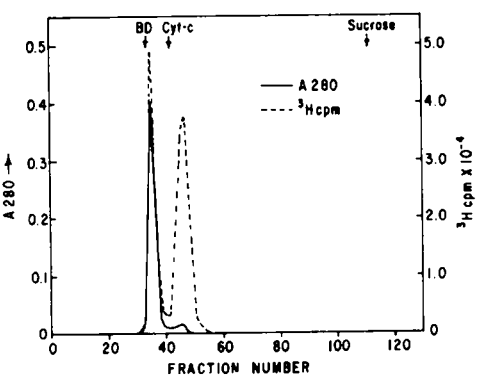
AMINO ACID ANALYSIS OF PROTEIN AND LIPID FRACTIONS FROM SODIUM DODECYL SULFATE CHROMATOGRAPHY

Values expressed as μmoles eluted in the total fraction for each peak. Values substituted by dash indicate the amino acid was not detectable; tr, indicates trace amount present.

Amino acid	*Protein fraction*	*Lipid fraction*
Lys	3.2	—
His	1.5	—
Arg	3.4	—
Asp	5.3	tr
Thr	3.4	tr
Ser	4.3	0.9
Glu	7.8	tr
Pro	2.9	—
Gly	3.6	tr
Ala	4.2	tr
Val	4.4	—
Met	—	—
Ile	3.0	—
Leu	7.1	—
Tyr	—	—
Phe	2.0	—
Ethanolamine	—	2.0

Chromatography in phenol–acetic acid–water or butanol–acetic acid–water

Separation of the protein and lipid of erythrocyte membranes can also be achieved by solubilization and chromatography in either phenol–acetic acid–water (1:1:1, by vol.) or butanol–acetic acid–water (1:1:1, by vol.). Fig. 4 shows the elution profile of the solubilized erythrocyte membranes from a Bio-Gel P-100 column using phenol–acetic acid–water as an eluent[19]. Included on the profile is the elution position of [^{14}C]cholesterol, which was used as a column marker. The elution position of the lipid peak indicates that it does not elute as an aggregate in this system. Amino



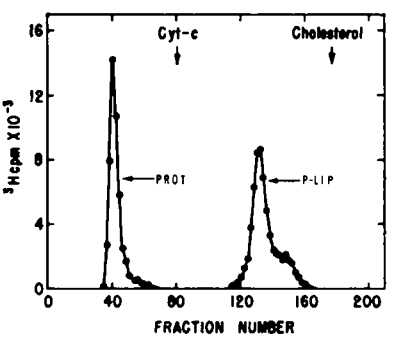


Fig. 3. Chromatographic profile of human erythrocyte membranes labeled with [^{3}H]acetic anhydride. Eluted from P-100 column (3.5 cm × 90 cm) with 1% sodium dodecyl sulfate. Void volume marked with Blue Dextran (BD). Protein (———) and radioactivity (-------).

Fig. 4. Chromatographic profile of labeled human erythrocyte membranes on P-100 with phenol–acetic acid–water. Void volume, Fraction 39. P-LIP = phospholipid.

acid analysis of the eluted fractions yielded results similar to those obtained with the sodium dodecyl sulfate column. Virtually all of the protein (> 95 %) was eluted in the first peak, while ethanolamine occurred only in the second peak.

Chromatography in butanol–acetic acid–water was performed on columns of Sephadex LH-20, since the columns prepared with Bio-Gel P-100 did not yield acceptable flow rates. Elution profiles of a sample of labeled human erythrocyte membranes are shown in Fig. 5 for an LH-20 column developed with butanol–acetic acid–water. The column was monitored for absorbance at 280 nm, ninhydrin-positive material and radioactivity. Amino acid analyses of column fractions were similar to those observed for the sodium dodecyl sulfate and phenol–acetic acid–water columns.

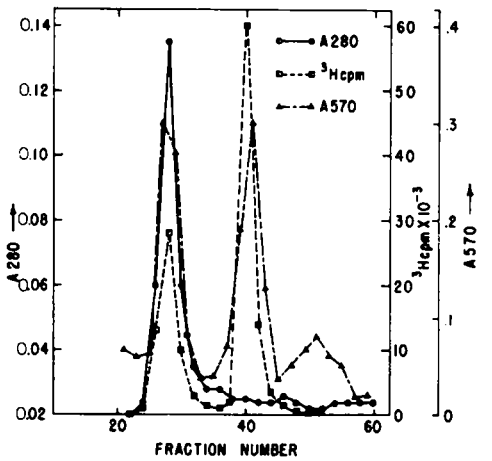


Fig. 5. Chromatographic profile of labeled human erythrocyte membranes eluted from LH-20 with butanol–acetic acid–water. Void volume, Fraction 26. Protein (———), ninhydrin reactive material (· · · · · ·) and radioactivity (- - - - - - -).

Solubilization and chromatography in cetyltrimethylammonium bromide–guanidine hydrochloride

The experiments of Gwynne and Tanford[20], in which about 60 % of the erythrocyte membrane was solubilized and fractionated in 6 M guanidine hydrochloride, led us to attempt to develop a system in which total solubilization of the membrane in high concentrations of guanidine could be achieved. Preliminary experiments showed that total solubilization (as measured by turbidity) was possible if the membranes were first treated with cetyltrimethylammonium bromide. Fractionation of the membrane was first attempted by chromatography on P-100 in 5.5 M guanidine hydrochloride. Only a single peak of radioactivity at the column void volume was obtained, indicating that no separation had been achieved. Analysis of the eluent after removal of guanidine by dialysis showed that both protein and phospholipid were still present. Because of the low resolving power of this column, the failure to separate the components could have resulted from fortuitous overlap of the protein peak with an aggregate of phospholipid. Therefore the cetyltrimethylammonium bromide–guanidine solubilized membrane was chromatographed on a 4 % Agarose column, which is known to fractionate the polypeptide chains of membranes which

are solubilized in guanidine hydrochloride solutions[20]. Once again only a single peak at the column void volume was eluted, and it contained both the protein and phospholipid of the membrane. This observation was also made with doubly labeled membranes. These experiments indicate that a soluble aggregate of membrane components must be formed in the presence of cetyltrimethylammonium bromide and guanidine hydrochloride. To show that this aggregate can be again fractionated into protein and lipid components, the guanidine and cetyltrimethylammonium bromide of the peak fraction from a P-100 column were removed by exhaustive dialysis. Solubilization and chromatography of the lyophilized residue on P-100 in 1 % sodium dodecyl sulfate gave the usual pattern of separated protein and lipid components similar to that shown in Fig. 3.

DISCUSSION

The question of the distribution of polypeptide chains in the erythrocyte membrane has received considerable attention in recent years. A number of different methods have been used to fractionate erythrocyte membranes into groups of components. These techniques include butanol[21] or pentanol[22] extraction, pyridine extraction and chromatography[23], chromatography in 2-chloroethanol[24], extraction and electrophoresis in acetic acid[25] and sequential extractions with EDTA, salt and organic solvents, followed by chromatography in sodium dodecyl sulfate[26]. Of particular interest are the reports of low molecular weight polypeptides as membrane components[6,18,27]. Both electrophoretic and chromatographic evidence has been presented in favor of the presence of low molecular weight polypeptides. Laico *et al.*[18] have suggested that a "miniprotein" represents a large fraction of the total membrane protein. In opposition to this concept, Fairbanks *et al.*[3] were not able to demonstrate such a component by electrophoresis, and Trayer *et al.*[28] did not find low molecular weight peptides in the proteins solubilized by guanidine hydrochloride. A complete description of membrane polypeptides may not be achieved by electrophoresis, however, since detection depends upon particular staining techniques. It is noteworthy that the membrane glycoprotein does not stain with common protein stains[3,29]. A full chromatographic analysis requires that all components of the membrane be solubilized and disaggregated.

Erythrocyte membranes have been solubilized and disaggregated in three different systems, 1 % sodium dodecyl sulfate, phenol–acetic acid–water and butanol–acetic acid–water. In each case the protein and the lipid of the membrane are disaggregated. In sodium dodecyl sulfate solution available evidence supports the contention that membrane proteins are disaggregated to the individual polypeptide chains[1,3,28]. The evidence is less secure in the two acetic acid systems, but electrophoretic studies by Demus and Mehl[30] in similar media indicate a high degree of polypeptide disaggregation. Further chromatographic studies to separate higher molecular weight components are necessary to characterize these systems more completely. Amino acid analysis of chromatographic fractions of the three different systems indicate that no significant fraction of the total membrane protein exists as a low molecular weight polypeptide. Less than 5 % of the protein was eluted after cytochrome *c* in each of the runs made in the different chromatographic systems.

It should be noted that attempts to measure protein by the Lowry method[31] gave higher protein values (20–25 % of total) for the lipid fraction of the sodium dodecyl sulfate eluent, indicating the poor reliability of this technique for measuring protein in the presence of lipid.

Examination of the chromatographic profiles shows that the state of the lipid varies in the different disaggregating media. In sodium dodecyl sulfate the lipid exists as a mixed aggregate (probably micellar) of detergent, phospholipid, cholesterol and glycolipid. It is this aggregate which appears to correspond to the "miniprotein" of Laico *et al.*[18], at least on electrophoresis. Phospholipids can be stained with Coomassie Blue on sodium dodecyl sulfate acrylamide electrophoresis gels, although the intensity of the stain is dependent on electrophoresis and staining conditions[8]. The chromatographic studies on labeled membranes indicate another area for misinterpretation. Both protein and aminophospholipids are labeled by most reagents that are attacked by nucleophiles. The failure to recognize this labeling of lipid probably contributed to the abnormally low molecular weight distribution of proteins reported by Kiehn and Holland[6]. Low molecular weight polypeptides can also arise from proteolytic digestion, which has been discussed in previous reports[3, 32].

Consideration of the separating abilities of the three fractionation methods suggests that they may be useful in several situations. Included among these are (1) separation of glycoprotein and glycolipid for individual carbohydrate analysis and determination of antigenic activities; (2)preliminary separation of protein and lipid before fractionation of protein components; and (3) separation of protein and lipid for recombination experiments, which permit the study of interaction of membrane proteins and lipids under different conditions. Preliminary experiments have indicated the applicability of the sodium dodecyl sulfate separation in each of these instances, and further experiments are underway to test the usefulness of the other systems.

The failure of the detergent–guanidine system to disaggregate the membrane proteins was somewhat surprising in view of the apparent solubilization of the entire membrane and the previous success of Gwynne and Tanford[20] in disaggregating a significant portion of the membrane protein. The results suggest that all of the membrane components are incorporated into large aggregates, which probably contain detergent as well. The current studies do emphasize the care which must be taken in extrapolating the use of membrane solubilizing agents to different systems. The solubilizing and disaggregating properties of guanidine are obviously sensitive to other components in the system and may even vary between different membrane systems without the introduction of other external agents such as cetyltrimethylammonium bromide. This indicates a further cautionary note which should be added to that of Maddy and Kelly[33] concerning the use of guanidine hydrochloride in membrane solubilization.

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REFERENCES

1 J. Lenard, *Biochemistry*, 9 (1970) 1129.
2 K. L. Carraway and D. Kobylka, *Biochim. Biophys. Acta*, 219 (1970) 238.
3 G. Fairbanks, T. L. Steck and D. F. H. Wallach, *Biochemistry*, 10 (1971) 2606.
4 C. A. Schnaitman, *J. Bacteriol.*, 104 (1970) 882.
5 C. A. Schnaitman, *J. Bacteriol.*, 104 (1970) 890.
6 E. D. Kiehn and J. J. Holland, *Biochemistry*, 9 (1970) 1729.
7 E. A. Grula and C. F. Savoy, *Biochem. Biophys. Res. Commun.*, 43 (1971) 325.
8 K. L. Carraway, A. Lam, D. Kobylka and J. W. Huggins, *Anal. Biochem.*, 45 (1972) 325.
9 D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, 30 (1958) 1190.
10 G. A. Bray, *Anal. Biochem.*, 1 (1960) 279.
11 S. Moore and W. H. Stein, *J. Biol. Chem.*, 211 (1954) 907.
12 J. H. Roe, *J. Biol. Chem.*, 212 (1955) 335.
13 J. T. Dodge, C. Mitchell and D. J. Hanahan, *Arch. Biochem. Biophys.*, 100 (1963) 119.
14 K. L. Carraway, D. Kobylka, J. Summers and C. A. Carraway, *Chem. Phys. Lipids*, 8 (1972) 65.
15 J. Philippot, *Biochim. Biophys. Acta*, 225 (1971) 201.
16 R. M. Zacharius, T. E. Zell, J. H. Morrison and J. J. Woodlock, *Anal. Biochem.*, 30 (1969) 148.
17 F. B. St. C. Palmer and J. A. Verpoorte, *Can. J. Biochem.*, 49 (1971) 337.
18 M. T. Laico, E. I. Ruoslahti, D. S. Papermaster and W. J. Dreyer, *Proc. Natl. Acad. Sci., U.S.*, 67 (1970) 120.
19 A. Pusztai and W. B. Watt, *Biochim. Biophys. Acta*, 214 (1970) 463.
20 J. T. Gwynne and C. Tanford, *J. Biol. Chem.*, 245 (1970) 3269.
21 A. H. Maddy, *Biochim. Biophys. Acta*, 88 (1964) 448.
22 R. F. A. Zwaal and L. L. M. van Deenen, *Biochim. Biophys. Acta*, 150 (1968) 323.
23 O. O. Blumenfeld, P. M. Gallop, C. Howe and L. T. Lee, *Biochim. Biophys. Acta*, 211 (1970) 109.
24 P. Zahler, *Vox Sang.*, 15 (1968) 81.
25 A. H. Maddy and P. G. Kelly, *FEBS Lett.*, 8 (1970) 341.
26 S. A. Rosenberg and G. Guidotti, *J. Biol. Chem.*, 244 (1969) 5118.
27 H. Glossman and F. Lutz, *Z. Physiol. Chem.*, 351 (1970) 1583.
28 H. R. Trayer, Y. Nozaki, J. A. Reynolds and C. Tanford, *J. Biol. Chem.*, 246 (1970) 4485.
29 K. L. Carraway, D. Kobylka and R. B. Triplett, *Biochim. Biophys. Acta*, 241 (1971) 934.
30 H. Demus and E. Mehl, *Biochim. Biophys. Acta*, 203 (1971) 241.
31 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 109.
32 D. Kobylka, A. Khettry, B. C. Shin and K. L. Carraway, *Arch. Biochem. Biophys.*, 148 (1972) 475.
33 A. H. Maddy and P. G. Kelly, *Biochim. Biophys. Acta*, 241 (1971) 114.