Membrane Proteins from *Bacillus megaterium* KM*

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ABSTRACT: A membrane protein fraction, with properties very similar to those of mitochondrial structural protein, and submitochondrial coupling factor F_4, has been prepared from cytoplasmic membranes of *Bacillus megaterium* KM by two different methods. This protein fraction, which is very insoluble at neutral pH, but soluble in alkali, appears homogeneous when dissolved in 0.25% sodium dodecyl sulfate, using ultracentrifugation and disc gel electrophoresis at pH 9.2 as criteria. However, when dissolved in a phenol–acetic acid–urea mixture the membrane protein fraction is inhomogeneous and on electrophoresis shows several strong bands and further weaker ones. The fraction binds lipids. Membrane protein fractions prepared by the two different methods show several common bands. A simple method of obtaining one of the major bands in the membrane protein fraction almost free of contaminants has been found. Polyacrylamide gel electrophoresis of whole cytoplasmic membranes reveals at least 12 clearly visible bands.

Some membranes from widely different sources contain similar functional systems. In particular, the cytoplasmic membrane of bacteria and the inner membrane of the mitochondrion both contain the electron transport chain and coupled energy production systems as an integral part of the membrane. We thought it would be interesting to see whether proteins similar to the structural protein fraction of mitochondria could be prepared from bacterial cytoplasmic membranes using similar preparative methods. *Bacillus megaterium* KM was chosen for this study because the cell wall can be simply and rapidly separated from the rest of the cell by use of lysozyme (Weibull, 1953) and the cytoplasmic membranes are then obtained free from cell wall and the soluble cell contents by lysing the protoplasts in a hypotonic solution. The bacterial cytoplasmic membrane has an advantage over mitochondria in investigating proteins of this type, since the single cytoplasmic membrane can be separated cleanly and easily from other cell components whereas mitochondria possess both inner and outer membranes which are not so easily separated. Recently there have been reports which indicate that mitochondrial structural protein prepared by the method of Richardson *et al.* (1964) is not homogeneous when prepared from beef heart mitochondria and contains at least six proteins (MacLennan and Tzagoloff, 1968). A preparation from liver yielded a preparation with fewer protein bands (Lenaz *et al.*, 1967). Allmann *et al.* (1967) report a purification of this preparation. Similarly the submitochondrial coupling factor F_4 which had been shown to have many similar properties to structural protein (Zalkin and Racker, 1965) is also composed of several different proteins, some of which seem identical with components from structural protein prepared by the method of Richardson *et al.* (1964).

We have prepared a membrane protein fraction from the cytoplasmic membrane of *B. megaterium* KM by methods similar to those used in preparing structural proteins from mitochondria, erythrocytes, and beef liver microsomes (Richardson *et al.*, 1964) and in preparing submitochondrial coupling factor F_4 from beef heart (Conover *et al.*, 1963). The properties of this protein fraction are very similar to the properties of mitochondrial structural protein fraction. Like mitochondrial structural proteins it is insoluble at neutral pH, in 8 M urea at pH 7.2, in 1% Triton X-100 at pH 7.2, but dissolves in 0.1% sodium dodecyl sulfate at pH 7.4 and in alkali in pH 11 (Criddle *et al.*, 1962). It also binds phospholipids and values for the sedimentation velocity coefficient of the main protein component when dissolved in sodium dodecyl sulfate at pH 7.4 are extremely close to values reported for both beef heart and yeast mitochondrial structural protein (Criddle *et al.*, 1962; Katoh and Sanukida, 1965). Like mitochondrial structural protein it is inhomogeneous when dissolved in phenol–acetic acid–2 M urea (MacLennan and Tzagoloff, 1968). We have also obtained one of the proteins in this fraction in a purified form.

Materials and Methods

Cultures of *B. megaterium* KM, originally obtained from Dr. R. Storck, were grown in 2% trypticase soy broth at 30° on a New Brunswick rotary shaker. Cells were harvested after overnight growth by centrifuging at 9000*g* for 10 min and were then washed twice with distilled water.

Preparation of Cytoplasmic Membranes. Each gram wet weight of whole cells was resuspended in 10 ml of 0.02 M phosphate buffer (pH 7.0) to which was added lysozyme (Sigma Grade 1) to a final concentration of 0.4 mg/ml of lysozyme, and DNase (Sigma DN-C) to a final concentration of 0.004 mg/ml. The cells were incubated at 25° until no intact organisms could be observed in several independent fields in the phase contrast microscope (usually 1–2 hr). At this stage lysis was

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judged complete and cytoplasmic membranes were collected by centrifuging at 35,000*g* for 1 hr. A white precipitate collects beneath the membrane at the base of the centrifuge tube (presumably β-hydroxybutyrate) and this is discarded. Alternatively the membranes were prepared by making protoplasts and then lysing, following the procedure of Broberg and Smith (1967). Before use membranes were washed at least four times with 0.01 M phosphate buffer (pH 7.0).

Preparation of Membrane Proteins. PREPARATION 1. This is a modification of the method used by Richardson *et al.* (1964) to prepare mitochondrial structural protein. A small modification was made by inserting a butanol extraction step after the membrane fragments had been dispersed in cholate–deoxycholate solution and centrifuged to remove insoluble material. Butanol extracts most of the yellow pigments of *B. megaterium* KM membranes so that contamination of the membrane proteins is minimized. Butanol is added to a concentration of 40% to a clear suspension of membranes in cholate–deoxycholate at 4°. This causes some precipitation of the membrane protein fraction. Further precipitation occurs when saturated ammonium sulfate (pH 7.1) is added to a final concentration of 12% (Richardson *et al.*, 1964). The butanol–aqueous phases are separated by low-speed centrifugation (9000*g* for 10 min) and at this stage the membrane protein fraction forms sheets which collect at the interface. The butanol is removed with a Pasteur pipet and the aqueous layer carefully poured off to leave the protein sheets on the walls of the centrifuge tube. From this stage on the procedure of Richardson *et al.* (1964) is followed exactly.

PREPARATION 2. This is based on the procedure of Conover *et al.* (1963) for the purification of submitochondrial factor F_4. Zalkin and Racker (1965) showed that F_4 was similar to mitochondrial structural protein prepared by the method of Richardson *et al.* (1964) and MacLennan and Tzagoloff (1968) showed that the two preparations have several proteins in common. The membranes are dissolved in 0.4 M ammonium hydroxide and then the structural protein is precipitated out at pH 8 with ammonium sulfate. We used only the fraction corresponding to Fraction A of Conover *et al.* (1963) and inserted a final wash with 90% acetone.

Protein was determined by a modification of the biuret method (Gornall *et al.*, 1949). Spectra were run on a Cary 14 spectrophotometer or a Zeiss PMQ II. Dry weight of membranes was determined by washing membranes very thoroughly with water and drying samples to constant weight at 105°.

Phospholipid Determination. We used Asolectin (Associated Concentrates) as a phospholipid source, preparing a suspension by the method of Conover *et al.* (1963). We also used the method of Mizushima *et al.* (1966) to obtain separate neutral and phospholipid fractions from cytoplasmic membranes of *B. megaterium* KM. Membrane protein fraction (5 mg) in 1 ml of 0.02 M Tris-HCl buffer was treated with about 13 mg of phospholipid suspension. After a 15-min incubation at room temperature the insoluble protein–lipid complex was removed by low-speed centrifugation and washed twice to remove excess lipid (Richardson *et al.*, 1963). Phospholipid was determined as total phosphorus by a modification of the procedure of Fiske and Subbarow (1925) and protein was determined by the biuret method. Some samples of membrane protein were dissolved at pH 11.2 in sodium hydroxide, the phospholipids were added, and after a suitable time the protein–phospholipid complex was precipitated by adjusting the pH of the solution to pH 7.

RNA Determination. A modification of the Schmidt–Thannhauser (1945) procedure suggested by Munro and Fleck (1966) was used.

Gel Electrophoresis. The system of Matson (1965) consisting of a 7% acrylamide gel with a running pH of 9.2 was used for some runs. We also adapted this system so that gels and sample solution contained 5.8 M urea. In general the membrane protein samples were dissolved in 0.25% sodium dodecyl sulfate–0.25 M sucrose before they were applied to the top of the gels. Gels were usually run for 14 min at a starting current of 2 mA/tube. The system of Takayama *et al.* (1966) in which samples are dissolved in a phenol–acetic acid–water (2:1:1, w/v) mixture containing 2 M urea and run in acetic acid–urea gels was used in other runs. Samples were run for 1.5 hr at a constant current of 5 mA/tube.

Ultracentrifuge runs were made at a speed of 56,100 rpm in a Spinco Model E ultracentrifuge using a synthetic boundary cell. Solutions were dissolved in 0.5% sodium dodecyl sulfate and phosphate buffer (pH 7.2). Mitochondrial structural protein was made from rat liver mitochondria and beef heart mitochondria using the method of Richardson *et al.* (1964).

Flagellin-Free Membranes. To ensure that the membrane protein fraction was not contaminated with flagellin we homogenized one preparation of cells for 1 min in a Waring Blendor before treatment with lysozyme (McDonough, 1965).

Results

Using the preparative methods of Richardson *et al.* (1964) and that of Conover *et al.* (1963) we obtained an almost white protein fraction which had properties very similar to those of mitochondrial structural protein and submitochondrial fraction F_4 of Racker. Only the fraction obtained under the same conditions as fraction A of Conover *et al.* (1963) had solubility and electrophoretic properties which were similar to those of the membrane protein fraction obtained by the method of Richardson *et al.* (1964). The slight yellow color of the protein is probably due to minor contamination with yellow pigments remaining in the cholate–deoxycholate phase after butanol extraction. The yellow pigments are associated with the neutral lipid fraction. The insoluble protein fraction comprises between 25 and 35% of the total cytoplasmic membrane protein when prepared by the method of Richardson *et al.* (1964) and contains only minor quantities of phospholipids (see Table I). The washed membranes contain an average of 65% protein by weight, which is in good agreement with the results of Yudkin (1966) who found about 70% protein in cytoplasmic membranes of *B. megaterium* KM. The

membrane protein fraction is insoluble at neutral pH, but dissolves in alkali at pH 11 and in 0.1% sodium dodecyl sulfate at pH 7 when freshly prepared. The preparation becomes much more insoluble on standing in suspension. The main protein components are insoluble in 8 M urea. It binds phospholipids (Table I) although the binding is less than that of mitochondrial structural protein (Richardson *et al.*, 1964). The ultraviolet absorption spectrum of the protein fraction dissolved in 0.25% sodium dodecyl sulfate shows a typical spectrum with a peak at 278 mμ, a shoulder at 290 mμ.

In the ultracentrifuge a solution of the protein prepared by the method of Richardson *et al.* (1964) and dissolved in 0.5% sodium dodecyl sulfate shows a single symmetrical peak although there is some material which sediments very rapidly to the bottom of the cell. The average sedimentation velocity constant of the single peak (three runs) was $s_{20,\omega} = 1.96 \pm 0.04$ S which compares very closely with the value of $s_{20,\omega} = 1.95$ obtained by Katoh and Sanukida (1965) for yeast mitochondrial structural protein and with the values of $s_{20,\omega} = 1.9$–2.2 found by Criddle *et al.* (1962) for beef heart mitochondrial structural protein and from which they estimated a molecular weight of 22,000 for the monomeric form of the proteins in sodium dodecyl sulfate.

Acrylamide gel electrophoresis at pH 9.2 (Matson, 1965) generally revealed two protein bands, one major and one minor, although in some preparations the minor band was not detectable (Figure 1). In some preparations there was a diffuse-staining band which moved ahead of the protein bands and which also stained with Sudan III and Rhodamine 6G, general lipid stains. This staining is largely attributable to the sodium dodecyl sulfate used to dissolve the protein and also to traces of cholate and deoxycholate which can remain associated with the protein even after acetone extraction. When a 0.25% sodium dodecyl sulfate solution is run alone in acrylamide an opaque region is found in the gels which stains less well than protein with Amido-Schwarz dye (Salton and Schmitt, 1967) and a similar staining can be found with cholate and deoxycholate. Even in samples which showed heavy stains of this type the phospholipid content was less than 1% by weight, assuming an average molecular weight of 668 for the phospholipids of the cytoplasmic membrane of *B. megaterium* KM. This molecular weight is based on the data of Yudkin (1966) who found that the main phospholipid in *B. megaterium* KM was phosphatidyl ethanolamine, and the main fatty acid constituents were 13-methyltetradecanoic acid (50–55%) and 12-methyltetradecanoic acid (25–30%). Salton and Schmitt (1967) have shown that any lipid present will move with sodium dodecyl sulfate on the gels. In general the preparative method of Conover *et al.* (1963) gave less diffuse staining but membrane protein yields were much lower usually between 10 and 12% of the total cytoplasmic protein. The main protein band moved through the gel at almost the same rate as the main band of mitochondrial structural protein. Preparations made by the methods of Richardson *et al.* (1964) and Conover *et al.* (1963) moved at the same rate through the gel

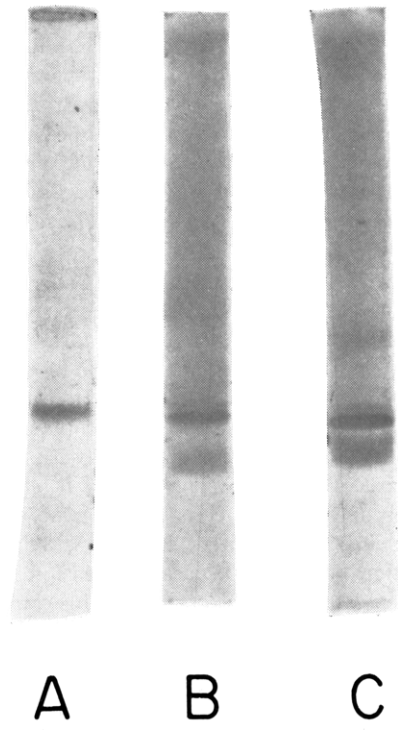

FIGURE 1: Polyacrylamide gel electrophoresis of membrane protein fractions, pH 9.2. (A) Membrane protein fraction, Conover method. (B) Membrane protein fraction, Richardson method, showing one main band, traces of second band, some diffuse staining in front of main protein band. (C) Mixture of protein fractions prepared by Richardson method and Conover method. Heavy diffuse staining in front of main protein band.

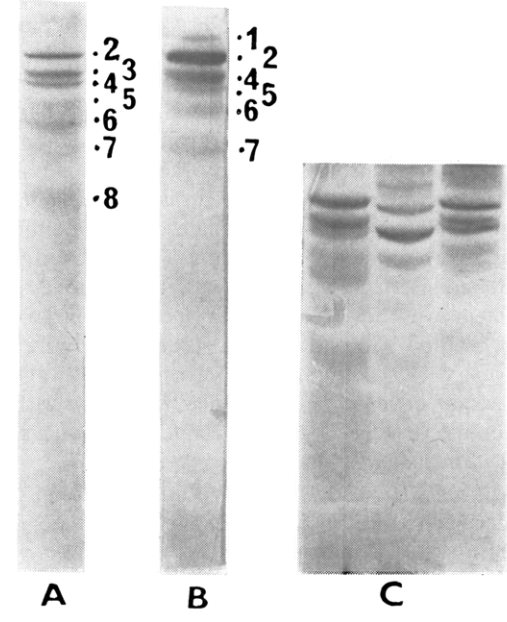

FIGURE 2: Polyacrylamide gel electrophoresis of membrane protein fractions, system of Takayama *et al.* (1966). (A) membrane protein fraction, Richardson method, showing seven bands. (B) Membrane protein fraction, Conover method, showing six bands. (C) Left to right. Richardson method; Conover method; mixture of Richardson method and Conover method.

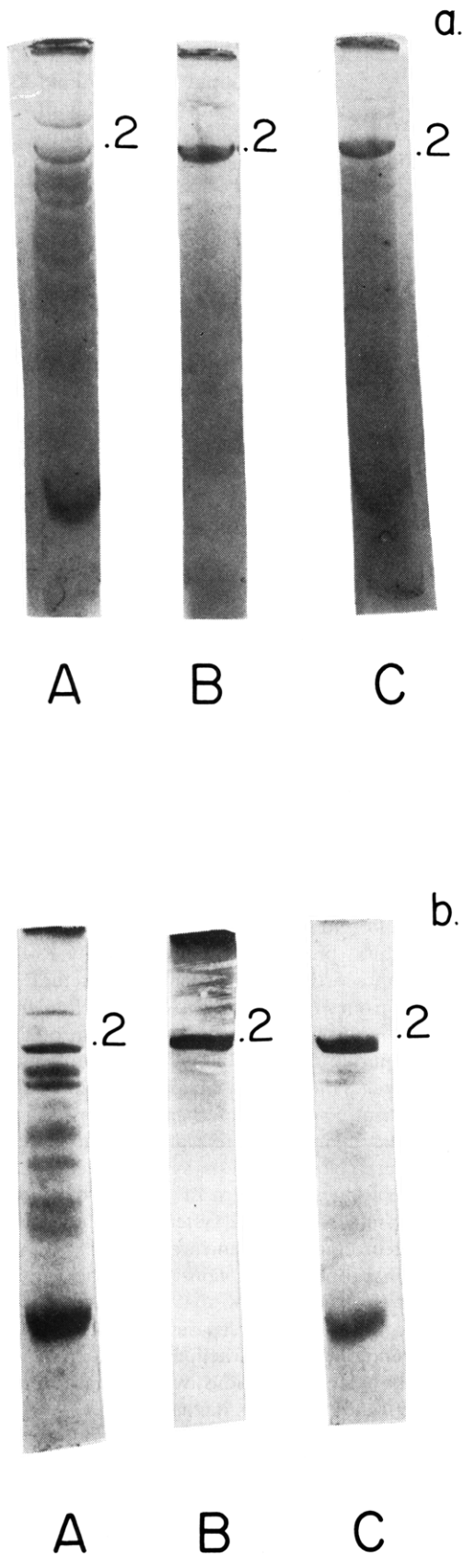

FIGURE 3: Polyacrylamide gel electrophoresis, system of Takayama *et al.* (1966). (A) Washed whole membrane of *B. megaterium* KM. (B) Purified preparation of band 2 of membrane protein fraction. (C) Mixture of A and B.

and when mixed together showed no additional separation of bands (Figure 1). The measured RNA content of our membrane preparations was less than 2% since the cells were lysed in magnesium free buffer, which results in low RNA concentrations in the cytoplasmic membrane, Schlessinger (1963). This low RNA content ensures that we are not looking at insoluble ribosomal proteins. Acrylamide gel electrophoresis of samples dissolved in 0.25% sodium dodecyl sulfate, 5.8 M urea, and run in 5.8 M urea gels still revealed one major band but there appeared to be several minor bands present with increased diffuse staining, in place of the one minor band seen when the protein is dissolved in sodium dodecyl sulfate alone. Mitochondrial structural protein prepared from rat liver mitochondria by the method of Richardson *et al.* (1964) also showed one major and one minor band when run at pH 9.2 in 0.25% sodium dodecyl sulfate.

The gel electrophoresis system of Takayama *et al.* (1966) greatly increased the resolution of the membrane protein fraction. Protein bands are numbered so that bands obtained on gel electrophoresis of one preparation correspond to those which are common to the other preparation (see Figure 2). At least seven distinct bands could be detected in preparation I made by the method of Richardson *et al.* (1964) which showed a maximum of two bands when dissolved in sodium dodecyl sulfate (Figure 2). Bands 2, 3, and 4 were most prominent. Preparation II, made by the method of Conover *et al.* (1963), showed six distinct bands, of which two were more prominent (bands 2 and 4) (Figure 2). Electrophoresis of mixtures of the two preparations revealed that all the protein bands of the second preparation except band 1 correspond to bands in the first preparation. Bands 2, 4, 5, 7, and 8 of preparation I correspond to bands, 2, 4, 5, 6, and 7 of preparation II respectively (Figure 2). Preparations made from four different batches of cytoplasmic membranes of *B. megaterium* KM showed the same band pattern.

Gel electrophoresis of whole cytoplasmic membranes using the method of Takayama *et al.* (1966) revealed at least 12 bands (see Figure 3) clearly visible in the gels. The bands were consistent between different batches of membranes. The front running band stained very diffusely was a slightly different color from the normal protein bands and looked opalescent when viewed against a dark background. It may not be protein. Both the procedure of Richardson *et al.* (1964) and that of Conover *et al.* (1963) effect a considerable purification of membrane proteins. However, we found that we could effect a much greater purification of one of the protein components of the membrane (band 2 in all preparations) by following the first step of the procedure of Conover *et al.* (1963). We dissolved the membranes in a mixture of 0.4 M ammonium hydroxide, 0.2 M potassium chloride, and 1 mM EDTA, centrifuged away insoluble material, and then brought the clear supernatant to pH 8 with acetic acid, when a precipitate formed on standing. This precipitate was collected by centrifugation at 10,000*g*/10 min. The yield is very low. Gel electrophoresis of this band in the Takayama system reveals one very heavy band with only minor con-

TABLE I: Binding of Asolectin to Membrane Protein from *B. megaterium* KM.[a]

Condn for Interaction of Membrane Protein	Time (min)	Temp (°C)	Phospholipid Added (μg of phosphate/mg of protein)	Phospholipid Bound (μg of phosphate/mg of protein)
A. Untreated membrane protein, pH 7.2, Tris-HCl buffer	a 15	25		0.35
	15	25	64	3.72
	b 15	25		0.51
	15	25	64	1.61
	c 15	25		0.5
	15	25	64	1.57
	15	25	128	1.61
B. Protein depolymerized at pH 11.3, phospholipid added, sedimented at pH 7	c 15	25	64	2.86
	15	25	128	4.21
	60	25	64	8.85
C. Purified protein, pH 7.2, Tris-HCl buffer	15			0.40
	15		64	3.3
	15		64	3.6

[a] Prepared by method of Richardson *et al.* (1964); a, b, and c are three different membrane protein preparations.

tamination from other membrane proteins. It is identical with band 2 of the *B. megaterium* KM cytoplasmic membrane, band 2 is one of the major components of the whole membrane (Figure 3). Lipid binding experiments with this protein give results which are within the range of those obtained with the membrane protein fraction prepared by the Richardson method (Table I).

Discussion

The membrane protein fraction isolated from cytoplasmic membrane of *B. megaterium* KM bears a strong resemblance to the structural protein fraction of mitochondria and to the submitochondrial coupling factor F_4.

Richardson *et al.* (1964) showed that the binding of phospholipid to mitochondrial structural protein was extremely sensitive to traces of cholate or deoxycholate associated with the protein and it is possible that the lower quantities of phospholipids bound by *B. megaterium* KM membrane proteins may be due to incomplete removal of the detergents. The phospholipid binding studies give no indication of which of the protein components is responsible for the binding although results with the purified protein from the membrane indicate that lipid binding capacity is similar to that of the less pure preparations. The inhomogeneity of the membrane preparations suggests detailed studies of phospholipid binding should await further purification of the membrane protein fraction in *B. megaterium* KM. Green *et al.* (1968) have pointed out that many of the data previously obtained on mitochondrial structural protein will have to be re-evaluated and our results agree very well with data from mitochondrial systems.

The use of the phenol–acetic acid–urea system to disperse the proteins before electrophoresis reveals that in the case of mitochondrial structural protein, submitochondrial coupling factor F_4 (MacLennan and Tzagoloff, 1968) and *B. megaterium* membrane proteins the fractions are not as homogeneous as at first they appeared to be. In *B. megaterium* KM there appear to be at least six protein components present under the more severe dispersal conditions of Takayama *et al.* (1966) two of which appear more prominent than the others. There is a report in a paper by Rottem and Razin (1967) that a structural protein fraction isolated by Rodwell with similar properties to mitochondrial structural protein isolated from *Mycoplasma laidlawii* which migrated as one band in the polyacrylamide gel system of Davis (1964) was split up into ten bands using the system of Takayama *et al.* (1966). Takayama *et al.* (1966) isolated four constituent complexes of the mitochondrial electron transport chain using the high resolution phenol–acetic acid–urea electrophoresis system. Each complex showed a reproducible pattern of many protein bands suggesting that the solvent system was not causing serious protein breakdown and was highly effective in separating hydrophobic proteins. Recent results of Tzagoloff *et al.* (1968) show that this electrophoresis system is highly effective in separating component proteins in structural protein fractions and submitochondrial coupling factor fractions which appear homogeneous by other criteria. Bagdasarian *et al.* (1964) give evidence that phenol–acetic acid–water has little hydrolytic activity toward proteins and peptides, but is highly effective in breaking hydrophobic interactions between protein units. The contrast between the multiple bands obtained in the electrophoresis system of Takayama *et al.* (1966) and the two or one bands obtained in sodium dodecyl sulfate at pH 9.2 and in the ultracentrifuge could be explained by the existence of a group of proteins of similar molecular weight and

hydrodynamic properties with similar charge at pH 9.2 and 7, but with very different charge at acid pH in the presence of phenol and urea. Our preliminary results with one protein component of the membrane, and the results of Allmann *et al.* (1967) in separating one component of the structural protein fraction of the mitochrondrial membrane suggest that it may be possible to separate the components with relative ease. The idea of a group of characteristic structural proteins which are common to a variety of membrane systems has recently been suggested by Green *et al.* (1968). It is recognized that the preparations obtained by these methods could contain functional proteins from the membrane. It seems unlikely that cytochromes or their apoproteins are present in significant quantity since the membrane preparations do not show absorption in the Soret region and the conditions used are unlikely to dissociate the hemoproteins to any great extent. Cholate and deoxycholate have been used routinely to solubilize mitochondrial membrane complexes without dissociation of heme groups from the cytochrome (Crane and Glenn, 1957).

The recent results with structural protein and submitochondrial coupling factor F_4 (Tzagoloff and MacLennan, 1968) and the results presented here suggest that the idea of a single structural protein comprising a large portion of the protein of membrane systems is much too simple, and at the very least there must be a class of proteins which serve the function of providing a basic structure for the membrane.

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