MICROCOCCUS LYSODEIKTICUS CELL MEMBRANE STRUCTURAL PROTEIN

By

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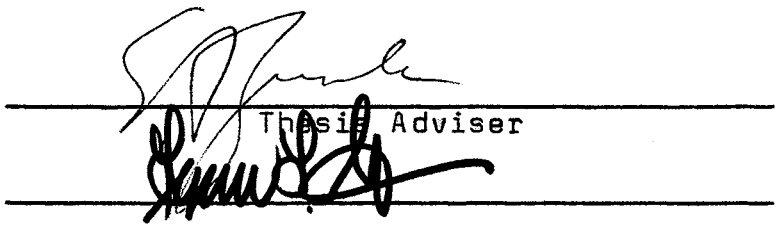

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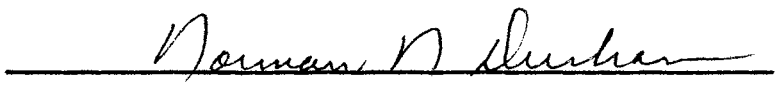

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CHAPTER I

INTRODUCTION

Electron microscopy has revealed the remarkable similarity in structure (characteristic triple-layered appearance, which is dark-light-dark) of cellular membranes, the endo-plasmic reticulum (cytomembranes), the outer, inner and cristae membranes of mitochondria, chloroplast membranes, the two membranes of the nuclear envelope, and the membranes of bacterial protoplasts and spheroplasts (Korn, 1966).

A similar mode of bonding may also be involved in holding membrane components together. Green et al. (1961) have summarized various reported phenomena now systematized as expressions predominately of the hydrophobic bond of mitochondrial membranes: the tendency of structural protein (S. P.) and cytochromes *a*, *b* and c_1 to form polymers at neutral pH when isolated and freed from other mitochondrial components; the monomerization of the cytochrome polymers by lipophilic reagents; the depolymerization of cytochromes *a*, *b* and c_1 by S.P.; the depolymerization of S.P. by the cytochromes; and the binding of lipid to S.P. and to the cytochromes. Razin, Morowitz and Terry (1965) speculated that the bonding mode in *Mycoplasma* (pleuro-

pneumonia-like organisms) membranes is apolar or hydrophobic. Butler, Smith and Grula (1967) presented evidence showing that the major mode of bonding in the membrane of Micrococcus lysodeikticus appears to involve hydrophobic groups.

The subunit concept, based on the premise that biological membranes are made up of elementary particles or subunits (Green and Hechter, 1965), has also been applied to both mitochondrial and bacterial membranes. Investigation has shown that membranes can be dissociated into what appears to be a subunit form and these can then be reconstituted in various ways. Green and Hechter state that these subunits are lipoprotein in nature, and possess both structural and functional roles in animal mitochondria.

Korn (1966) summarized a number of studies, which indicate that homogeneous lipoprotein subunits may be formed by disaggregating biological membranes in certain detergent solutions. Among the studies mentioned were those of Razin et al. (1967), who found that the surface membrane of Mycoplasma laidlawii disaggregated in dodecyl sulfate solution to give a lipoprotein subunit having a homogeneous size as evidenced by a single schlieren peak of about 3 S in the Model E ultracentrifuge. Similarly, Salton and Netschey (1965) have disaggregated membranes from M. lysodeikticus and Sarcina lutea with detergent solutions, and obtained fractions defined as homogeneous after ultracentrifugation. Bovine myelin (Huldher, 1953), rat myelin

(Gent et al., 1964) and erythrocyte membranes (Bakerman, 1965) have also been disaggregated to give subunits reported to be homogeneous as judged by disc gel electrophoresis as well as sedimentation behavior in the Model E ultracentrifuge.

Contrary to the idea that detergent treatment of biological membranes results in the formation of homogeneous lipoprotein subunits, Engelman, Terry and Morowitz (1967) showed, by use of density-gradient centrifugation, that a high degree of separation of lipid and protein occurs in membranes treated with 10 mM sodium dodecyl sulfate. Because a single well-defined schlieren peak was obtained during ultracentrifugation, they questioned whether or not ultracentrifugation and the schlieren patterns obtained are sufficiently rigid criteria to establish presence or absence of homogeneity in such complex systems.

Rottem, Stein and Razin (1968) augmented the findings of Morowitz and his co-workers by reporting that disaggregation of the Mycoplasma membrane by sodium dodecyl sulfate separates membrane lipid from protein as demonstrated by polyacrylamide gel electrophoresis of the disaggregated material. From studies of schlieren patterns, they also observed that membrane protein and lipid had similar patterns, and when mixed together, gave a single symmetrical peak. They concluded that the Model E analytical ultracentrifuge is unable to separate protein from lipid when disaggregation has been effected in sodium dodecyl sulfate.

Also contrary to the subunit concept are the findings reported by Grula and Savoy (1971). They found, by polyacrylamide gel electrophoresis, that the membrane of M. lysodeikticus as well as the envelopes (cell wall plus membrane) of Serratia marcescens and Erwinia species are very complex, with the membrane of M. lysodeikticus resolving into more components than previously described using the Model E ultracentrifuge (Butler et al., 1967). These data, in addition to the observations of Engelman et al. (1967) and Rottem et al. (1968), indicate that the subunit concept, as previously described, is probably not applicable to bacterial membranes.

The ability of membranes to undergo disaggregation and subsequent reaggregation of the component parts is another similarity evidenced in different bacterial membranes. Razin et al. (1965) reported that the peripheral membrane of M. laidlawii can be disaggregated by treatment with detergent and, under conditions of dialysis, reaggregated into a membranous structure. Dialysis was performed in a solution containing a monovalent salt (sodium chloride), a divalent cation (magnesium ions) and a reducing compound (B-mercaptoethanol). Experimentation revealed that in order for membranous structure formation to occur, the detergent must be removed and a divalent cation must be present.

Rottem et al. (1968) also demonstrated the need for a divalent cation during dialysis to induce aggregation. They reported that although monovalent cations permit some

unstable reaggregation, presumably by neutralizing the negatively charged groups present on lipid and protein molecules, only divalent cations give rise to a stable structure since they are able to form salt bridges between the negatively charged molecules.

Since M. laidlawii does not contain a cell wall, it was not known whether the cell membrane from a cell wall-containing organism would also aggregate under similar conditions of dialysis until the report of Butler et al. (1967). These investigators demonstrated that the cell membrane of a Gram-positive organism, M. lysodeikticus, could be readily disaggregated using an anionic (but not a non-ionic or cationic) detergent such as sodium lauryl sulfate (SLS). They also reported that reaggregation of the disaggregated membrane components into ordered membranous structures resembling the original membrane, both chemically and structurally, occurs during dialysis in a menstruum of low ionic strength and in the presence of a divalent (but not trivalent) cation such as magnesium, calcium or manganese ions at near neutral pH. Although conditions for aggregation were similar to those reported for Mycoplasma, neither monovalent salt nor reducing agent were essential.

Other aspects of the aggregation phenomenon were also reported by this group (Butler et al., 1967; Grula et al., 1967). They showed that the amount of aggregation was directly proportional to temperature, at least up to 37° C. A difference also appeared to exist between whole and ag-

gregated membranes in that the latter were disaggregated to a significantly greater extent by two cationic detergents (G-271 and G-3436A). This was thought to indicate a possible structural difference. In addition, a relationship was observed between the magnesium ion concentration and pH as to their effect on aggregation. Aggregates formed at pH 3.5 appeared to require less magnesium than aggregates formed at pH 7.4. This was, however, attributed to the acid pH, which was thought to cause precipitation of the disaggregated membrane components.

This group also tested whole and aggregated membranes to determine the type of bonding involved in holding the membrane components together. Treatment with urea, guanidine, ethylenediaminetetraacetic acid (EDTA), or incubation at various pH values (from 3.0 to 10.0) did not cause disaggregation. The only chemical agents found to cause significant disaggregation were bifunctional detergent molecules, preferably anionic. Like Mycoplasma, the major mode of bonding in M. lysodeikticus membranes appears to involve hydrophobic groups.

From studies of the membrane protein and lipid association during aggregation, it was also found that membrane proteins, stripped of phospholipids and carotenoid pigments, could be disaggregated, then reaggregated into ordered membranous structures without addition of the stripped materials. Electron microscopy of a cross section of this type of aggregate showed the characteristic triple-layered

structure. These findings indicate that membrane structuring as seen using the electron microscope is probably due to protein associations. It was concluded that lipids, although present in the intact membrane, are not necessary for proper orientation of the protein molecules. It was also suggested that instead of a protein-lipid-protein type structuring for membranes (the protein "sandwich" of Danielli and Davson, 1935), the basal membrane continuum consists of protein-to-protein interactions with lipid associated with the top and bottom of these molecules (lipid-protein-lipid structuring).

From studies of protein and lipid association in mitochondrial membranes, Fleischer et al. (1962) concluded that membrane architecture is preserved in particles stripped of lipids. Contrary to this conclusion, Green et al. (1967) stated that stripped mitochondrial membrane subunits cannot aggregate to form ordered membrane sheets. Instead, the stripped subunits appear to precipitate. Based on their experiments, they concluded that phospholipids are essential for ordered aggregation.

To further elucidate events occurring during aggregation and, also, to better understand membrane structuring, Rice (1969) studied the aggregation phenomenon using disaggregated components from the envelope of Erwinia species, a Gram-negative bacterium. He found that the envelope could be disaggregated by SLS, and reaggregated into membranous sheets during dialysis in the presence of magnesium

ions.

Rice further observed that the amount of aggregation was dependent upon the magnesium ion concentration and pH of the dialysis menstruum. He reported that an increase in magnesium ion concentration increased the total amount of aggregation as well as the number of proteins present in the aggregates. Utilizing this information, a partial purification of the major protein species (thought to be S.P.) present in the envelope complex of Erwinia species was accomplished by dialysis at pH 9.1 using a limited concentration of magnesium ions (0.001 M).

Structural protein was first reported as a major protein constituent of heavy beef heart mitochondria by Green et al. (1961). Later, Green et al. (1968) advanced the idea that noncatalytic proteins are major constituents of all sectors of mitochondrial membrane repeating units.

Most investigations which relate to possible existence of S.P. have been accomplished using animal mitochondria wherein the issue is far from settled. Only two reports have appeared which relate to bacterial membranes. In one of these studies (Mirsky, 1969), it was concluded that no single species of S.P. exists in the cell membrane of Bacillus megaterium KM; at the very least, there must be a class of proteins which serve the function of providing a basic structure for the membrane. Data obtained using Bacillus PP, a mutant of B. megaterium KM (Patterson and Lennarz, 1970), may lead to an opposing conclusion since a

single species of protein, having a molecular weight of 32,000, comprises at least ninety percent of the total protein in the cell membrane of this mutant. In the parent organism, the same protein exists as a relatively minor component.

The studies reported in this thesis were undertaken to determine if a single species of protein could be obtained from the membrane of M. lysodeikticus which would aggregate to form ordered membrane sheets. Although testing would be possible, the assumption was made that ordered aggregation, or assembly into membranous sheets, could not occur using either denatured or unlike molecules. Thus, ordered aggregation can be viewed as a specific morphological type of biological assay for S.P.. If evidence for such a single species of protein could be obtained, it was hoped that further studies regarding chemical characteristics could be accomplished.

CHAPTER II

MATERIALS AND METHODS

Cell Growth And Membrane Isolation

Cells of Micrococcus lysodeikticus were grown in a defined medium (Grula, 1962), and membranes isolated by the technique described by Butler, Smith and Grula (1967).

Membrane Disaggregation and Reaggregation

Unless otherwise specified, buffer used was always 0.0025 M Tris (pH 7.4), and all centrifugations were accomplished at 54,500 x g for thirty minutes using the Spinco Model L centrifuge.

After isolation, membrane preparations were washed three times in cold Tris buffer, resuspended in 9 ml of the same buffer and 1 ml 0.2 M SLS added (0.02 M final concentration) to disaggregate the membranes. After thirty minutes at room temperature, the disaggregated membrane components were separated from all detergent insoluble materials by centrifugation. The clear, yellow supernatant was placed at 4° C overnight to precipitate most of the SLS which was then removed by centrifugation.

To permit reaggregation to occur, the concentration of disaggregated membrane components was adjusted using

Tris buffer to an optical density (O.D.) of 0.19 - 0.2 at 440 nm, placed in dialysis tubing, and dialyzed using a volume ratio of 1:120 (inside-outside) at 26° - 28° C. Initially, dialysis was performed only in 0.0025 M Tris buffer. This buffer was changed after twelve hours, and dialysis continued an additional twenty-four hours in the presence of 0.01 M magnesium chloride unless otherwise specified. Dialysis in the absence of magnesium ions further aids in removal of SLS not precipitated at 4° C. At the end of the dialyzing period, the aggregated material was sedimented by centrifugation, washed three times using cold Tris buffer, and stored at -30° C, after being freeze-dried (lyophilized), prior to further processing.

Membrane Stripping

To extract or remove most of the phospholipids and carotenoid pigments (stripping), lyophilized membrane material was extracted for one hour at 4° C using a cold solution of acetone, concentrated ammonium hydroxide and water (90 ml:50 ul:10 ml). Acetone and ammonium hydroxide were mixed immediately before addition to the membrane suspension (1 mg per ml H_2O), which was continually stirred during the addition and extraction. After stripping, the remaining membrane material was sedimented and washed twice in cold Tris buffer. For further study, the materials removed by stripping (stripping materials) were concentrated under reduced pressure at a temperature of 30° C.

Polyacrylamide Gel Electrophoresis

Detergent Gel System

Detergent (SLS) gel electrophoresis was performed in 10% acrylamide gels utilizing the basic systems of Davis (1964), Ornstein (1964) and Shapiro, Vinuela and Maizel (1967) as described and further refined by Weber and Osborn (1969) and Grula and Savoy (1971).

Stock solution A contained 7.8 g $NaH_2PO_4 \cdot H_2O$, 38.6 g $Na_2HPO_4 \cdot 7H_2O$ and 2 g SLS per liter. Stock solution B contained 11.1 g acrylamide and 300 mg N,N'-methylenebisacrylamide (BIS) per 50 ml. Insoluble materials were always removed from the solutions by filtration through Whatman No. 1 filter paper. Stock solution A was kept at 37° C. Stock solution B was kept at room temperature, and stored in the dark. Both stock solutions were prepared fresh every two weeks.

The glass tubes used to prepare the gel columns were 75 mm long having an inner diameter of 8.5 mm: one end was covered with a double layer of Parafilm during gel preparation. Before use the tubes were cleaned with acid cleaning solution, rinsed in distilled water (three times), soaked in Photo-flo cleaning solution (one drop per 100 ml H_2O), rinsed again (three times) and oven dried (100° C for twenty minutes).

For electrophoresis of a set of gels (12), 15 ml of stock solution A and 13.5 ml of stock solution B were mixed.

After five minutes, 1.5 ml of freshly prepared ammonium persulfate solution (8 mg per ml H_2O) and 50 ul N,N,N',N'-tetramethylenediamine (TEMED) were added. The resulting solution was then mixed and about 1 ml immediately placed into each of the 12 tubes using a 5 ml syringe. A few drops of distilled water were carefully layered on top of the gel columns before the acrylamide polymerized (usually within ten to fifteen minutes after the final mixing). The gels were kept at room temperature for at least five hours or overnight for complete polymerization before electrophoresis was attempted. Just prior to electrophoresis, the water and Parafilm were removed.

Protein samples were solubilized (3 - 4 mg per ml) at 37° C for two hours in solvent buffer (pH 7.1) containing 78 mg $NaH_2PO_4 \cdot H_2O$, 386 mg $Na_2HPO_4 \cdot 7H_2O$ and 1 g SLS per 99 ml distilled water. Immediately before use, 1 ml B-mercaptoethanol was added per 100 ml of solvent.

Glycerol (three drops per ml solvent buffer) was added just before samples (30 - 60 ul volumes) were applied to the tops of the gels. After the samples had been applied, the remainder of each tube was carefully filled with stock solution A (diluted 1:1 with distilled water). The two buffer chambers were filled with this diluted solution, and electrophoresis was performed at room temperature (six to seven hours) using 5 mA/tube with the anode located in the lower chamber. Buffalo Black NBR (Napthol Blue Black) in glycerol (3 mg per ml) served as the marker

dye (2 ul per tube).

After electrophoresis, the gels were stained in Coomassie Brilliant Blue (CBB) overnight at room temperature (1.25 g CBB in a solution containing 454 ml 50% methanol and 46 ml glacial acetic acid). The gels were then rinsed in distilled water two times prior to being transferred to destaining solution (75 ml glacial acetic acid, 50 ml methanol and 875 ml H_2O). Destaining was accomplished at 37°C on a reciprocal shaker with a change of destaining solution every half hour. After four hours, the gels were further destained in 8% acetic acid, then stored in distilled water in the dark.

Non-Detergent Gel System

Non-detergent gel electrophoresis was performed utilizing the pH 2.7 system described by Neville (1967) except that a stacking gel was not used. The gels are composed of 7% acrylamide, 0.2% BIS, 0.58 M acetic acid (3.5 %), 0.0075 M KOH and 9 M urea. Polymerization is catalyzed by the use of the following chemicals: 0.0004% riboflavin, 0.05% ammonium persulfate and 0.08% TEMED.

The working solution (stock solution A) contained 7 g acrylamide, 0.2 g BIS, 16.3 mg KOH, 54 g urea and 3.5 ml glacial acetic acid in a final volume of 100 ml, and was stored in the dark at room temperature for as long as three weeks. The catalytic solution (stock solution B) was always prepared fresh and contained 100 mg riboflavin

and 2.2 g ammonium persulfate per 100 ml water.

To prepare one set of gels, 21.5 ml stock solution A, 0.5 ml stock solution B and 50 ul TEMED were mixed and immediately placed in the tubes. A few drops of acetic acid (3.5%) were then carefully layered over the top of the mixture, and the tubes left overnight at room temperature under a fluorescent light to insure complete polymerization.

Buffer chambers (upper and lower) of the electrophoretic apparatus were filled with 10% acetic acid (Takayama et al., 1966), and the lower electrode served as the cathode. Electrophoresis was carried out with cold running tap water flowing through the jacket of the apparatus for forty minutes with a constant current of 2.5 mA/tube.

To avoid the known anomalies caused by exposure of proteins to persulfate (Mitchell, 1967), gels were pre-electrophoresed (5 mA/tube for one hour) to remove persulfate. After this pre-running step, the chambers were emptied and refilled with freshly prepared 10% acetic acid.

Protein samples were solubilized (3 - 5 mg per ml) in phenol, acetic acid and water (2:1:1, W/V/V; Takayama et al., 1966) at 25° C for two hours. For some experiments, urea (2 M) was also added to the solvent. Sample solutions (60 - 70 ul volumes) were applied to the tops of the gels and the remainder of the tube was carefully filled with electrophoretic buffer (10% acetic acid).

After electrophoresis, the gels were stained for one hour in 7% acetic acid containing Buffalo Black NBR (1%).

Destaining was accomplished in 8% acetic acid on a reciprocal shaker at 37° C with a change of destaining solution every half hour until the gels were destained (usually about twelve changes). Gels were stored in distilled water in the dark.

Densitometer Tracing

Polyacrylamide gel electropherograms (detergent and non-detergent) were made using a Canalco Model K Densitometer. For CBB staining, a yellow filter (Wratten #15, peak transmittance 560 nm) was utilized.

Phospholipid Staining

Staining of phospholipids was accomplished using Oil Red O stain. It was prepared by first saturating warm 100% methanol with Oil Red O and then making the methanol 70% by adding hot distilled water. The precipitate, which formed on cooling, was removed by filtration (0.45 μ millipore filter).

Gels were stained for six hours or overnight, and destained in 8% acetic acid on a reciprocal shaker at 37° C with a change in destaining solution every half hour until destained (usually about eight changes). Storage was in distilled water in the dark.

Glycoprotein Staining

Glycoprotein staining was performed utilizing the

procedure described by Zacharius et al. (1969), with the exception that after electrophoresis gels were kept in 12.5% trichloroacetic acid overnight (or twelve hours) followed by washing in distilled water (100 - 150 ml per gel) for an additional twelve hours on a reciprocal shaker prior to staining. Gels were stored in distilled water in the dark.

Iron (Ferrous) Stain

Analysis for iron in membrane samples was performed utilizing 1,10-phenanthroline (O-phenanthroline). The reduced form of iron (ferrous) when complexed with 1,10-phenanthroline gives an intense red complex color (absorbing at 512 nm), which can be used for colorimetric determinations of iron in the range of a few parts per million of solution. The iron (ferric) phenanthroline complex is pale blue and not of sufficient intensity for colorimetric estimations.

Lyophilized samples (less than 2 mg per ml final reaction volume) were solubilized in 4.4 ml SLS electrophoretic solvent, 0.2 ml β-mercaptoethanol added to insure reduction of the iron, and 0.4 ml 0.1% 1,10-phenanthroline solution added to test for reduced iron. After thorough mixing, the mixture was incubated at 37° C for six hours, and optical density readings made at 512 nm. Calibration curves were constructed utilizing known amounts of $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$.

Detection on SLS polyacrylamide gels was performed by two methods. (1) Samples, reduced and complexed with 1,10-phenanthroline as previously described, were electrophoresed, and the gels immersed in 0.1% 1,10-phenanthroline solution. Densitometer scanning (yellow filter) was immediately performed since color is not stable. (2) Samples were solubilized and electrophoresed as described in the procedure for detergent electrophoresis, and gels were immersed in 0.1% 1,10-phenanthroline solution. After allowing the gels to sit in the solution for fifteen minutes, densitometer scanning was performed. The latter method is the least desirable because the iron (ferrous) phenanthroline complex is soluble and distinct peaks are difficult to obtain.

Ammonium Sulfate Fractionation

In addition to the use of polyacrylamide gel electrophoresis, membrane proteins were also fractionated utilizing various amounts of ammonium sulfate. This procedure was accomplished at 4°C with the desired ammonium sulfate concentration determined from the nomogram of Di Jeso (1968).

Thirty mg of lyophilized, stripped membrane aggregate (prepared by stripping aggregates formed at pH 9.0 in the presence of 0.01 M magnesium ion concentration) were resuspended in 50 ml 0.05 M Tris buffer (pH 9.0) and disaggregated by the addition of SLS (0.02 M final concentration). The clear, slightly yellow membrane solution was

then placed at 4° C overnight to precipitate residual SLS.

Ammonium sulfate concentrations of 4% (2.5 g/100 ml), 8% (5 g/100 ml) and 25% (14 g/100 ml) were used and prepared by dissolving the compound in cold 0.05 M Tris buffer (pH 9.0) to a final volume of 50 ml. After SLS had been removed from the membrane solution, the two solutions (ammonium sulfate and membrane) were poured together, mixed thoroughly, and then placed at 4° C for three hours to allow precipitation. Precipitated membrane proteins were sedimented by centrifugation and residual ammonium sulfate, present with the precipitate, was removed by dialysis against 0.0025 M Tris buffer (pH 9.0) at room temperature using a volume ratio of 1:120 (inside-outside). This buffer solution was changed three times at two hour intervals.

Electron Microscopy

Washed membrane samples were resuspended in distilled water and placed on collodion-coated 400 mesh copper grids. This material was allowed to sit for thirty seconds, after which the excess was drawn off using filter paper, The material was then negatively stained by adding a drop of 2% uranyl acetate to the grid. After thirty seconds contact, the uranyl acetate was also drawn off using filter paper, and the grid allowed to dry. Specimens were examined using an RCA EMU-3G electron microscope at an accelerating voltage of 100 Kv.

Cystine-Cysteine Determination

Performic Acid Oxidation

Determination of cystine-cysteine was accomplished by oxidizing these compounds to cysteic acid (Moore, 1963). To 10 mg lyophilized membrane, 2 ml performic acid solution (one part 30% hydrogen peroxide and nine parts 88% performic acid) was added. This mixture was kept near 0° C (ice bath) for four hours to allow complete solubilization of the protein and oxidation to cysteic acid. To avoid over oxidation and subsequent loss of cysteic acid, excess performic acid was destroyed by adding 0.3 ml 48% HBr, with constant swirling of the reaction tube in the ice bath.

Concentration of the reaction mixture and removal of bromine formed were accomplished at 40° C under reduced pressure (rotary evaporator in a water bath). Twenty ml 1 N NaOH were added to the rotary condenser to absorb the bromine. Concentration of the reaction mixture to dryness took approximately thirty minutes.

Cysteic Acid Analysis

Analysis for cysteic acid in protein hydrolyzates was accomplished utilizing two-dimensional paper and thin-layer chromatography (TLC). The solvent systems contained tert-butyl alcohol, concentrated HCl and water (70:6.7:23.3, V/V/V) in the first direction and isopropyl alcohol, formic acid and water (70:10:20, V/V/V) in the second di-

rection (Abelson et al., 1957). Detection was accomplished by spraying with ninhydrin (0.05% in 95% acetone) followed by heating for thirty minutes at 65° C.

Amino Acid Analyses

Quantitative analyses were performed using the Beckman Model 120 C amino analyzer in the Department of Biochemistry under the supervision of Dr. P. E. Guire.

For qualitative analysis, samples were first stripped then hydrolyzed in 6 N HCl at 105° C for eighteen to twenty-four hours *in vacuo*. Hydrolyzates were concentrated to dryness under a warm flow of air from a hair dryer, solubilized in a small volume of deionized water, and then spotted either on Whatman No. 1 filter paper or TLC plates (MN-300 Cellulose). Solvent systems employed to resolve the amino acids were those of Heathcote and Jones (1965) or Redfield (1953). Detection was accomplished by spraying with ninhydrin as given above.

Radioisotope Labeling and Liquid Scintillation Counting

Cells were grown in defined medium, and radioisotopes (diluted to the desired concentrations with non-labeled carrier) were added after twelve hours of growth. The following radioisotopes were utilized: Acetate-2-^{14}C (0.002 uC/ml), L-aspartate-U-^{14}C (0.004 uC/ml), L-glutamate-U-^{14}C (0.0025 uC/ml) and glycerol-1-^{14}C (0.005 uC/ml). Liquid

scintillation counting of ^{14}C-labeled material was performed (1 ml sample in 9 ml counting solution) using Aquasol (New England Nuclear Company, Boston, Massachusetts).

CHAPTER III

RESULTS

Membrane Isolation and Analysis

In this study of the membrane of M. lysodeikticus, the established techniques of Butler et al. (1967) were employed for cell growth, membrane isolation and disaggregation (solubilization). Experiments were monitored utilizing electron microscopy (direct observation of deposited suspensions), electrophoretic analysis (polyacrylamide gel) and, when considered necessary, measurement of light absorption by carotenoid pigments at 440 nm.

The appearance of an isolated whole membrane (Figure 1) represents an organized (ordered) structure. When a similar appearing structure was fabricated during aggregation, we chose to refer to the structure as an "ordered" membrane sheet. Such sheets differ from unordered aggregates in that the latter possess various shapes, are usually of small size and appear to be thicker (compare Figures 1, 15 and 17). The presence or absence of ordered sheets served as a specific morphological type of biological assay in all aggregation experiments.

To determine the number of proteins necessary for formation of ordered membrane sheets, we planned to analyze

aggregated membranes utilizing polyacrylamide gel electrophoresis. Unfortunately the advent of electrophoretic techniques into biological studies has only been recent, and exact relationships and effects of each system on different biological materials are not totally understood. There are two basic systems into which all gel systems can be classified, i.e., detergent and non-detergent.

Since my problem required examination of all proteins present in whole as well as various types of membrane aggregates, the technique of polyacrylamide disc gel electrophoresis, most sensitive for this type analysis, had to be utilized. It was first necessary, therefore, to test various systems of electrophoresis in order to gain information concerning which system would be best suited for the analyses I would be attempting.

Non-Detergent Gel Electrophoresis

Survey of electrophoretic systems utilized by investigators studying membrane protein composition revealed three generalities. (1) Non-detergent type systems were in use several years before detergent types; only recently have detergent systems been developed. (2) The number of non-detergent type systems is very large. (3) Nearly all data reported utilizing non-detergent electrophoretic systems were obtained using gel systems that were modified in various ways. For this reason, it is difficult, if not impossible, to critically analyze and compare data. As a

result, investigators often choose to utilize several different gel systems.

Neville (1967) utilized two acid (pH 4.3 and 2.3) and one basic (pH 9.4) gel systems in an attempt to fractionate the proteins of liver cell plasma membranes. At that time, these gel systems were considered to be standard types for electrophoresis. Neville further reported, however, that none of these systems adequately resolved the membrane proteins; therefore, he modified the pH 2.3 gel system. Utilizing his modified system (pH 2.7), he was able to fractionate the plasma membrane into at least fifteen protein species. Primarily because of this report, we began this study using his pH 2.7 gel system.

Our choice seemed wise because Okuda and Weinbaum (1968) and also Mirsky (1969) later published membrane protein fractionation data, which they obtained utilizing both acid and basic gel systems. Even though Okuda and Weinbaum studied the envelope proteins of Escherichia coli and Mirsky the membrane proteins of B. megaterium KM, both reached the same conclusion, i.e., more protein species can be resolved using an acid gel system.

Analysis using the pH 2.7 gel system shows that at least nineteen protein species are present in the membrane of M. lysodeikticus (Figure 2). To obtain a pattern of sufficient intensity for analysis, however, it was necessary to deposit large amounts of membrane (approximately 250 ug) on top of the gel columns. Further, it was observ-

ed that gel patterns from the same or different membrane preparations were seldom reproducible. Thus, it appeared either that protein aggregation was occurring during electrophoresis or many proteins were not entering the gels due to incomplete solubilization.

During an electrophoretic experiment, a casual observation convinced us that solubility of proteins was responsible, in large measure, for most of the problems encountered using the acid system. Many of the proteins, which appear to be solubilized in the Takayama et al. (1966) solvent, precipitate and stack on top of the gel columns as soon as current is applied and mixing of solvent and buffer occurs. The result of stacking is shown at the top of the gel column in Figure 3A, and is observed after staining and destaining. This behavior of membrane proteins can also be demonstrated by adding proteins solubilized in the Takayama solvent to electrophoretic buffer (10% acetic acid) in a test tube wherein a lot of precipitation occurs immediately on mixing.

Attempts to compare membrane protein patterns I obtained with those published by Salton, Schmitt and Trefts (1967) were futile even though I used the same organism and electrophoretic system that they utilized. Their pictures show that only ten or eleven proteins are present, and the one of greatest intensity is located at or near the gel front. They described this particular protein as a 'fast running' protein and implied that it is important in

membrane structuring. A similar observation was made in our earlier studies; however, we concluded that this is an anomalous effect of ammonium persulfate, as described by Mitchell (1967), since it can be remedied by pre-electrophoresis of the gels to remove persulfate.

In summary, I observed that electrophoretic analysis of membrane proteins in an acid gel system yielded data that were not satisfactory in that stacking of proteins on top of the gels occurred, results were not reproducible and changes in migration of proteins occurred due to some type of interaction with ammonium persulfate. Because of these difficulties, polyacrylamide gel systems which utilize detergent for solubilization and as a part of the buffer system were screened. Detergent systems seemed to be a logical choice since previous membrane aggregation studies accomplished in our laboratory utilized SLS to solubilize membranes (Butler et al., 1967).

Detergent Gel Electrophoresis

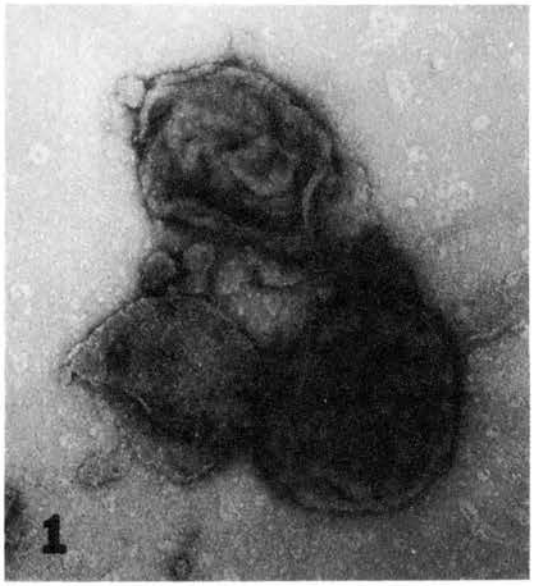
Several detergent systems were tested and evaluated; the one described by Weber and Osborn (1969) was found to be the most desirable. They demonstrated that the system was reliable in that protein patterns were reproducible and molecular weights (MW) of individual proteins could be determined (forty proteins of known MW were tested with little deviation in the range from 10,000 to 100,000 daltons).

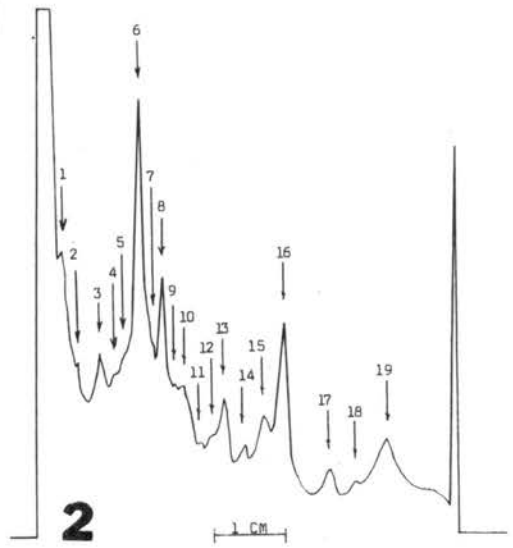
Figure 1. Isolated whole membrane negatively stained with uranyl acetate (60,000X).

Figure 2. Electropherogram pattern (acid gel system) of isolated whole membrane.

Figure 3. Electrophoretic gel columns containing resolved whole membrane components
A. Acid gel system (240 ug membranes)
B. SLS gel system (120 ug membranes)

Figure 4. Electropherogram patterns (SLS gel system) of different isolated whole membrane preparations
A. 120 ug of sample
B. 80 ug of sample


1


1
2
3
4
5
6
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10
11
12
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14
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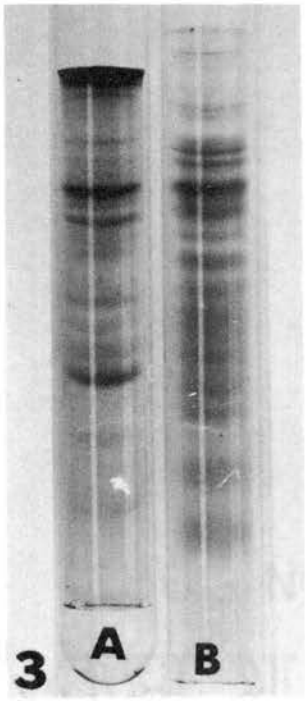
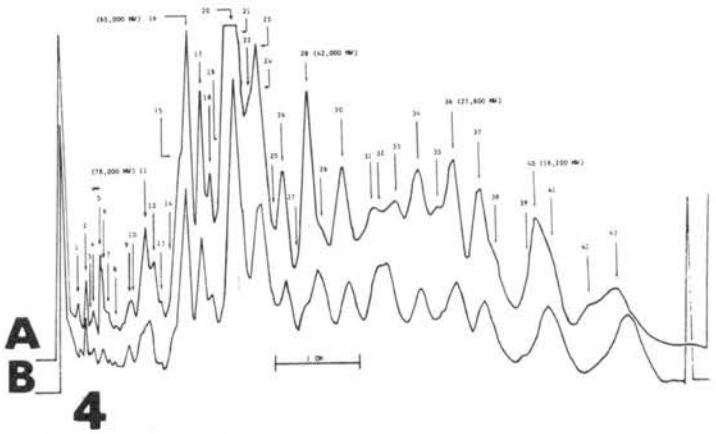
16
17
18
19
1 CM
2


A
B
3


A
B
4

The desirability of using Weber and Osborn's system in this study was further enhanced when it was found that anomalies due to ammonium persulfate do not occur (membrane protein patterns are identical in the presence or absence of persulfate).

Electrophoresis using the SLS gel system reveals that at least forty-three protein species are present in the membrane of M. lysodeikticus (Figures 3B and 4A,B). The mere fact that this number is more than double the number (nineteen) of species resolved in the acid (pH 2.7) gel system (Figures 2 and 3A) demonstrates the greater sensitivity of the SLS gel system. Further, the reliability of the SLS gel system is demonstrated by the reproducibility of the membrane protein patterns shown in Figure 4A,B. The two electropherogram patterns were obtained from different membrane preparations, which were separately analyzed. A picture of a representative gel and protein pattern using each type system is shown in Figure 3A,B. It can readily be seen that little, if any, stacking occurs in the SLS gel system.

To determine the MW of the membrane proteins resolved in the SLS gel system, proteins of known MW were electrophoresed, and a MW calibration curve constructed (Figure 5). I found the MW curve to be reproducible; however, lysozyme always had a mobility rate slower than expected. This behavior was also reported by Shapiro, Vinuela and Maizel (1967). They stated that they did not understand

Figure 5. Distance traveled by proteins of known MW in a detergent gel column. BSA = Bovine serum albumin, LDH = lactic dehydrogenase, DNase I = deoxyribonuclease and Cyto C = cytochrome c from horse heart

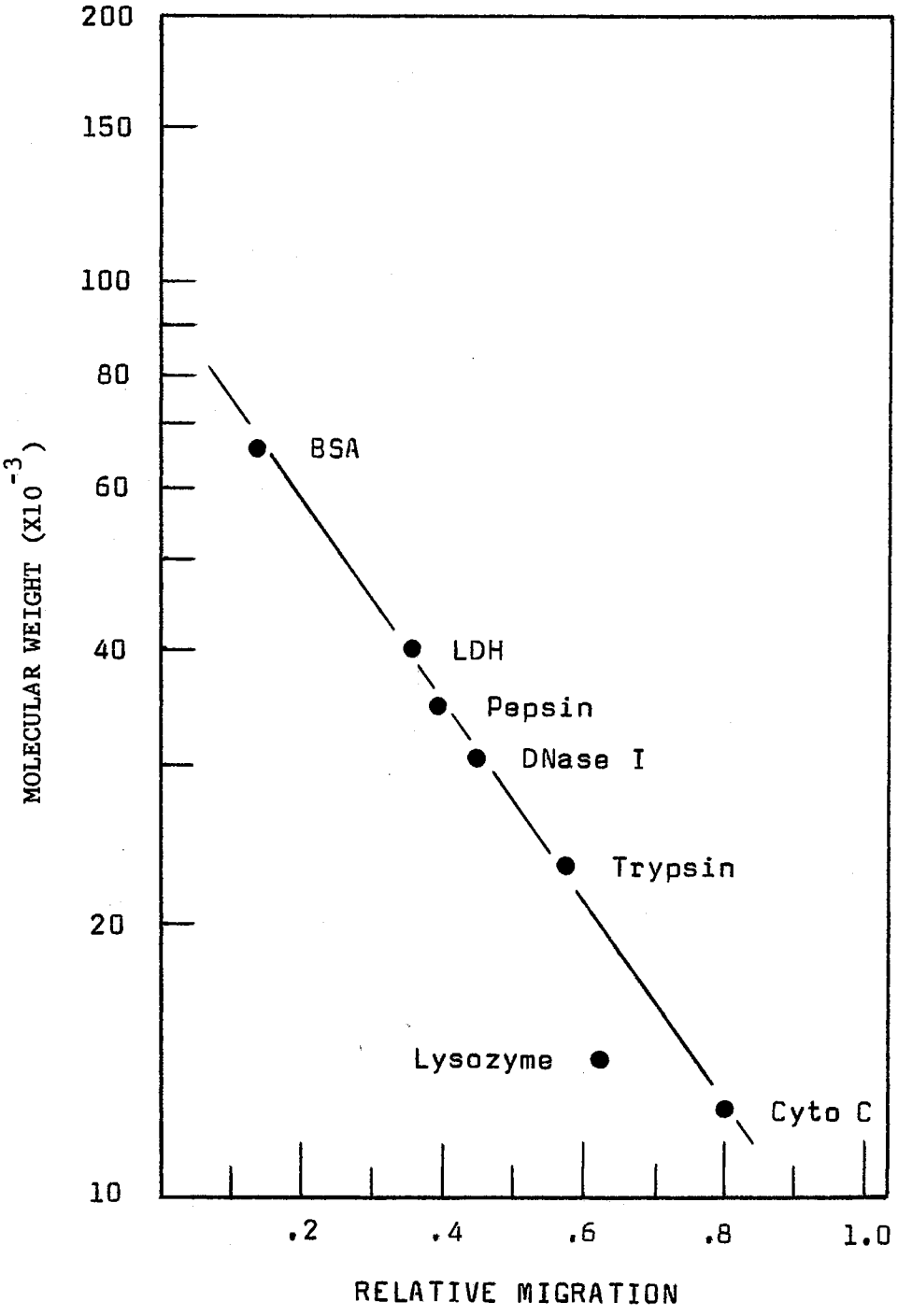

200
150
100
80
60
40
20
10
MOLECULAR WEIGHT (X10^{-3})
BSA
LDH
Pepsin
DNase I
Trypsin
Lysozyme
Cyto C
.2
.4
.6
.8
1.0
RELATIVE MIGRATION

TABLE I

MOLECULAR WEIGHT ESTIMATION OF THE MEMBRANE PROTEINS OF *MICROCOCCUS LYSODEIKTICUS*

Protein Species Number*	Molecular Weight (daltons) Exp. #1	Exp. #2	Exp. #3	Average
1	100,200	97,000	101,000	99,400
2	100,000	95,000	100,000	97,300
3	98,000	93,000	98,500	96,500
4	97,500	92,000	97,000	95,500
5-6	96,000	89,000	94,000	93,000
7	93,000	86,000	92,500	90,500
8	91,000	85,000	90,300	87,700
9	86,000	83,500	87,500	85,600
10	84,000	81,500	85,000	83,500
11	78,000	77,000	79,000	78,000
12	75,500	75,500	76,500	75,800
13	72,000	73,500	75,000	73,500
14	70,000	70,600	72,000	71,000
15	64,500	68,000	69,000	67,200
16	61,500	67,000	67,000	65,200
17	59,000	62,500	62,000	61,200
18	53,500	58,000	59,500	57,000
19	52,000	57,500	57,300	55,600
20	50,000	56,000	55,500	53,800
23	47,500	52,500	52,000	50,700
26	45,000	48,000	47,000	46,700
28	41,500	44,500	42,500	42,800
29	36,500	42,000	41,000	40,000
30	32,500	38,000	36,500	35,700
31-32	30,500	37,000	33,500	33,700
33	28,000	31,500	31,000	30,200
34	27,500	31,500	29,000	29,200
36	26,400	30,500	26,500	27,800
37	24,000	27,000	23,500	25,000
38	22,200	25,300	21,000	22,800
39	17,000	24,200	17,000	19,400
40	14,000	19,500	15,000	16,200
43	12,000	14,300	12,400	12,900

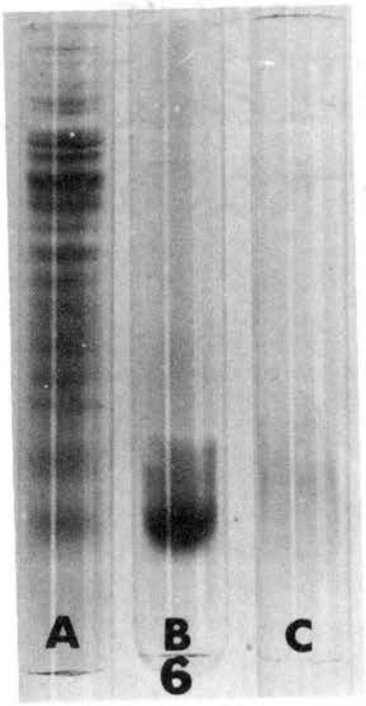
*Some protein species are not given because they are not completely resolved in gels.

the atypical mobility of lysozyme molecules, and thus far, we have not ascertained a reason for such behavior either.

As shown in Table I, the majority of the membrane protein species have MW in the range from 20,000 to 100,000 daltons. Since little, if any, material remains at the top of the SLS gels, it appears that none of the proteins present in the cell membrane of M. lysodeikticus have MW greater than 100,000 daltons. The species with MW less than 20,000 daltons are difficult to resolve since they often migrate as diffuse bands and deviate as much as twenty-five percent from the average MW (none, however, run ahead of the tracking dye). In addition, it was observed that after CBB staining, species 42 and 43 had colors (shades of blue) other than the blue color typically observed with the other protein species. This indicates that these proteins may exist as complexes with lipids and/or carbohydrates.

During electrophoresis, carotenoid pigments migrate to the same gel area as proteins 42 and 43 (Figure 6A). It was also observed that the area of the gel to which the carotenoids migrate can be stained with CBB. Two different components present in stripping materials, however, appear in this area after staining with CBB; one is a darkly stained fast-running component (species 43) which migrates directly ahead of the less intensely stained component (Figure 6B). To determine if these are phospholipids, selective staining utilizing Oil Red O was performed

Figure 6. Electrophoretic gel columns (SLS gel system) containing resolved membrane materials.
A. Isolated whole membrane (120 ug) stained with CBB.
B. Stripping materials stained with CBB.
C. Stripping materials stained with Oil Red O.


A
B
C
6

on another gel also containing stripping materials. It was found that only the gel area corresponding to the less intensely CBB-stained component, described above, stained an orange color (Figure 6C), and thus, is most likely composed of phospholipids. Since the 'faster running' CBB-stained component does not stain with Oil Red O, it seems reasonable to conclude that it is a protein which can be partially removed from membrane materials by stripping. This information indicates that either a detergent-resistant lipoprotein complex exists in the membrane of M. lysodeikticus or protein species 43 and membrane phospholipids migrate in a similar manner in our SLS gel system.

Of particular interest was our observation that protein species 43 has a MW of about 12,900 daltons (Table I). This is very close to the MW (13,400 daltons) reported by Atlas and Farker (1956) for cytochrome c isolated from horse heart. In addition, it has been reported that cytochromes of the a, b and c type are present in M. lysodeikticus (Salton and Ehtisham-ud-din, 1965) and firmly attached to the membrane (Nachbar and Salton, 1970). This information indicates that protein species 43 could be a cytochrome of the c type.

Since cytochromes always contain iron, I thought that this could possibly be selectively demonstrated by devising a test that would demonstrate the presence of iron molecules. Ayers (1958) reported that the reduced form of iron, when complexed with 1,10-phenanthroline, gives an in-

tense red color. The test is very sensitive and can be used for colorimetric determinations of iron in the range of a few parts per million of solution. One of the first experiments was a colorimetric determination for the presence of iron in the membrane of M. lysodeikticus; data are given in Table II.

Several points can be made concerning the test and the results obtained. (1) The test appears to be valid in that cytochrome c (contains iron) exhibits an increase in optical density after reduction and addition of 1,10-phenanthroline, even though it initially absorbs strongly at 512 nm. Further, trypsin (iron not present in molecule) does not absorb appreciably at 512 nm either before or after reduction and addition of 1,10-phenanthroline. (2) Testing has been performed using whole membrane samples isolated from different preparations of cells, and the concentration of iron (ferrous) was found to be approximately 1.25 ug per mg dry weight in all tests. Membrane samples also absorb at 512 nm before reduction; however, unlike the situation that exists using cytochrome c, this absorption is minimal. Periodic readings during incubation revealed that, using cytochrome c, maximum absorption was reached within two hours whereas, using whole membranes, maximum absorption was not reached for six hours. Because samples appear to be solubilized in the SLS electrophoretic solvent, this information suggests that iron molecules are not immediately dissociated from membrane components upon

TABLE II

DETERMINATION OF IRON (Fe^{++}) IN THE MEMBRANE OF *MICROCOCCUS LYSODEIKTICUS*

Sample Tested	Concentration (mg/ml)	O.D. at 512 nm*** Before	O.D. at 512 nm*** After	ug Fe^{++}/mg Sample****
Cytochrome c	0.19	0.14	0.20	0.93
Cytochrome c	0.36	0.26	0.36	1.05
Trypsin	0.22	0.00	0.02	0.00
Whole Membrane Sample #1*	0.20	0.05	0.27	1.21
Whole Membrane Sample #2	0.10	0.04	0.15	1.28
Whole Membrane Sample #3	0.24	0.06	0.31	1.18
Whole Membrane Sample #4	0.50	0.09	0.62	1.22
Whole Membrane Sample #5**	0.34	0.08	0.53	1.70

*Whole membrane sample was prepared separately from other membrane samples.

**Whole membrane sample was stripped prior to iron determination.

***Readings made before and after addition of 1,10-phenanthroline.

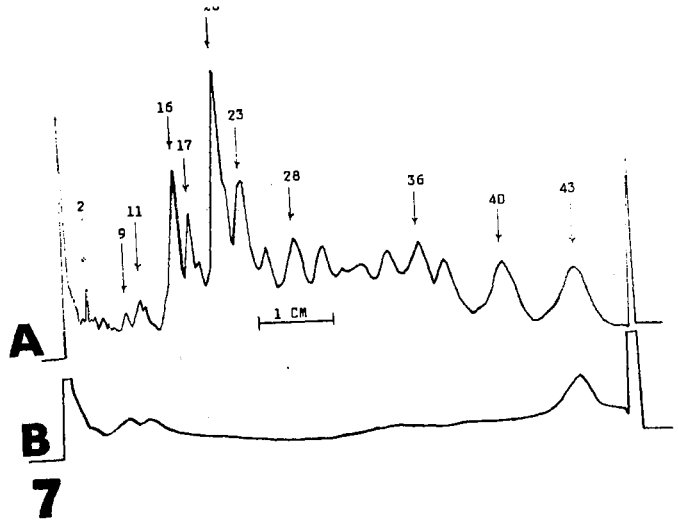
****Quantitation based on standard curve made using $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$.

disaggregation. Rather, it appears that a gradual release of iron occurs, followed by subsequent reduction and color formation with 1,10-phenanthroline.

Since whole membrane material can be disaggregated without immediate dissociation of iron from the membrane component(s), it seemed reasonable that the phenanthroline test could be utilized in SLS gel electrophoretic experiments to determine which of the protein species are associated with iron. In one type of experiment, membrane samples were solubilized, reduced and 1,10-phenanthroline added prior to electrophoresis. Duplicate samples were also electrophoresed; however, these proteins were not pretreated with the 1,10-phenanthroline reagent. After electrophoresis, both types of gels were immersed in 0.1% 1,10-phenanthroline solution.

Proteins in the samples complexed with 1,10-phenanthroline prior to electrophoresis were found to be more intensely stained than those from samples complexed only after electrophoresis. Regardless of when treatment with phenanthroline is accomplished, it can be observed that staining is concentrated in two areas, i.e., two peaks located in the MW range from 75,000 to 90,000 daltons and a peak with a mobility rate identical or almost identical to the rate of protein species 43 (Figure 7A,B). As shown, most of the staining is concentrated in the peak corresponding to species 43, and can readily be seen by visual observation of 1,10-phenanthroline-stained gels.

Figure 7. Electropherogram patterns (SLS gel system) obtained from isolated whole membrane samples treated with:
A. CBB (120 ug membranes)
B. 1,10-phenanthroline reagent (180 ug membranes)


2
9
11
16
17
23
28
36
40
43
1 CM
A
B
7

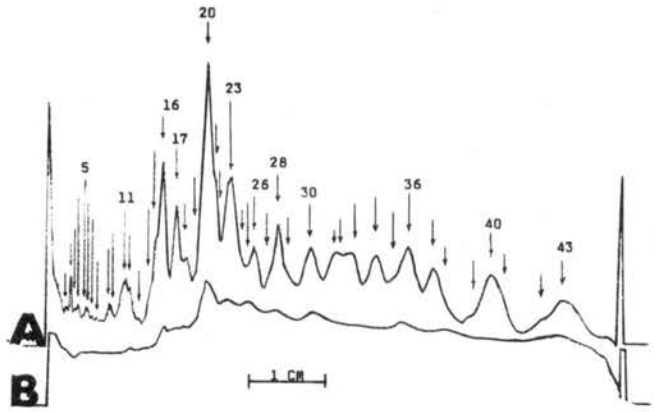
To summarize data pertaining to the membrane component species 43, it was found that: (1) it is partly protein; (2) the protein is closely associated with and may actually be bonded to phospholipids; (3) it is partially removed from the membrane by stripping; (4) iron is present; (5) the complex has a MW of approximately 12,900 daltons; and (6) it migrates with cytochrome c when co-electrophoresed on the same gel. Consequently, it seems reasonable to conclude that protein species 43 in the membrane of M. lysodeikticus is a cytochrome of the c type.

In addition to study of membrane materials by staining or complexing with CBB, Oil Red O and 1,10-phenanthroline, staining for glycoprotein was also done. Gilby, Few and McQuillen (1958) have reported the presence of mannose and possibly galactose in membrane preparations from M. lysodeikticus. MacFarlane (1964) reported that mannose in the membrane of this organism is present as a homopolysaccharide, mannan.

Our results indicate that several proteins, most in the MW range from 30,000 to 65,000 daltons, appear to be complexed or closely associated with carbohydrate material (Figure 8A,B). The species most obviously associated appear to be 20, 23, 26, 28 and 30. In addition, there seems to be some association between species 43 (cytochrome c) and carbohydrate material. The carbohydrate may, however, be associated with phospholipid rather than with species 43. Since it was found that phospholipid (stripping mater-

Figure 8. Electropherogram pattern (SLS gel system) of isolated whole membrane samples (120 ug) treated with:
A. CBB
B. Glycoprotein stain

Figure 9. Electrophoretic gel columns (SLS gel system) containing resolved stripping materials treated with:
A. Glycoprotein stain
B. CBB



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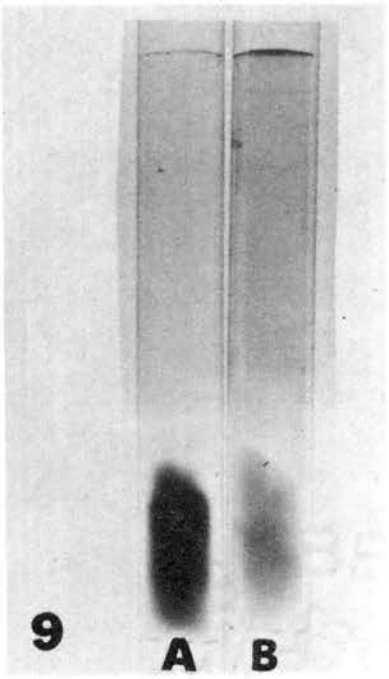


9

ials from membrane materials) is stained by this procedure (Figure 9A,B). Such a result would not be unexpected since it has been demonstrated that mannolipids are present in the membrane of this organism (Lennarz and Talama, 1966).

Utilizing E. coli and ^{14}C-labeled D-glucosamine, Okuda and Weinbaum (1968) have been the only investigators to report the presence of glycoproteins in the peripheral structures of bacteria; however, they were not able to distinguish between cell wall and membrane proteins. To aid in determining specificity of the glycoprotein staining procedure, lysozyme, L-alpha-cephalin (dipalmitoyl form), tripalmitin and several carbohydrate-containing proteins (pepsin, ovalbumin and red blood cell membrane components) were separately electrophoresed and stained using the glycoprotein stain. It was found that only the carbohydrate-containing proteins give a positive test as glycoproteins.

Although further study utilizing known glycoproteins should be done, it seems reasonable to conclude that, in addition to glycolipids and a homopolymer of mannose, some carbohydrate is present in the membrane of M. lysodeikticus complexed with protein and can therefore be classed as glycoprotein.

To determine if further dissociation of proteins or peptides could be accomplished prior to or during electrophoresis, several modifications of the detergent gel system were attempted.

(a) To dissociate peptides held together by divalent

metals, ethylenediamine tetraacetic acid (EDTA) was added (0.001% in the solvent and gel complex and 0.01% in the buffer). No further increase in number of proteins resolved occurred.

(b) To break disulfide bonding, β-mercaptoethanol was incorporated into the gel system (1% in both the solvent and gel complex). Although this agent does not bring about further dissociation of proteins, I did observe that resolution is enhanced (sharper banding) with the reducing agent present. For this reason, β-mercaptoethanol has been incorporated into the solvent for all electrophoretic analyses.

(c) To dissociate ionically bonded peptides, pH of the gel system (solvent, buffer and gel complex) was changed from pH 7.1 - 7.2 to 8.5 with and without EDTA present (as described above). Further dissociation of proteins does not occur.

(d) Hydrogen bonding: Reaggregation of disaggregated membrane components will occur in the presence of 6 - 8 M urea (evidence to be presented later). This allows the conclusion that incorporation of urea into the SLS gel system will not cause further dissociation of the proteins. In addition, it has been reported that incorporation of 8 M urea into the gel complex (Keihn and Holland, 1968) or prior treatment of membrane samples using urea (Schnaitman, 1969) does not increase protein dissociation in SLS gels. Thus, I did not attempt to further dissociate the

membrane proteins by incorporating urea into the SLS gel system.

(e) Heat treatment: It was found that neither heating the samples in the solvent (boiling water bath for fifteen minutes) nor heating the gel columns during electrophoresis (accomplished by running a mixture of hot and cold water through the electrophoretic apparatus jacket at approximately 50° C) increases protein dissociation. It was observed, however, that the resulting electropherogram patterns (obtained under both conditions) were less intense than usual and protein was stacked on top of the gels. This latter observation indicates that heat causes some denaturation which results in a decrease in sample solubility.

(f) Treatment by stripping: By selective staining using Oil Red O, it was found that, for the most part, phospholipids are separately resolved in SLS gels from the various protein species. Nevertheless, stripping and subsequent electrophoresis of the preparations was performed to determine if stripping allows increased dissociation of membrane proteins. Because the resulting gel pattern was observed to be like those shown in Figures 3B and 4A,B, it may be concluded that stripping does not allow further dissociation of membrane proteins. This conclusion is in agreement with Patterson and Lennarz (1970), who reported that the protein pattern obtained from Bacillus PP was unaffected by prior stripping.

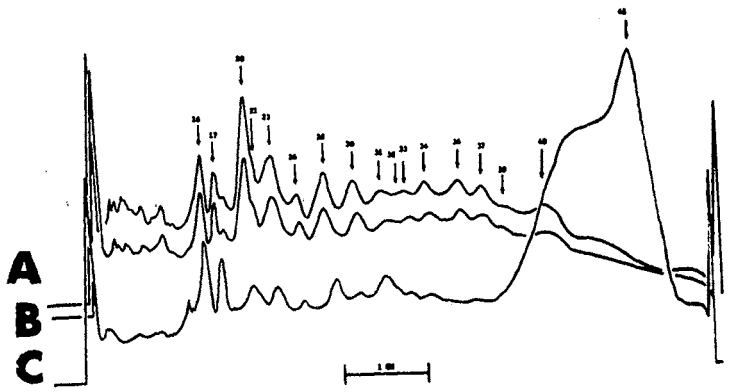
Even though it was observed that stripping does not

appear to alter the membrane protein patterns, two pertinent questions still needed to be answered. (1) Are any membrane proteins removed by stripping? (2) If proteins were found to be removed by stripping, was removal in any way selective?

To answer these questions, 20 mg of lyophilized whole membrane were stripped and the stripping materials concentrated to dryness as previously described. The material was then dissolved in 1 ml electrophoretic solvent (SLS system), and 80 ul amounts (on three different gels) electrophoresed. In addition, different amounts of non-stripped whole membrane sample (30, 60, 90, 120 and 150 ug) were also electrophoresed for visual standards. If proteins are removed by stripping, it was thought that the amount of protein removed could be estimated by comparing the intensity of protein stains on the gels to those in the visual standards. Data given in Figure 10A,B,C reveal that some proteins are removed by stripping; however, the total amount in 80 ul of concentrated stripping material is a little less than that obtained from 30 ug of non-stripped whole membrane.

Using figures reported by Gilby et al. (1958) for composition of the cell membrane of M. lysodeikticus (protein, 50%; phospholipids, 28%; and carbohydrate, 15 - 22%), it can be concluded that 30 ug of non-stripped whole membrane contain about 15 ug protein. The percent of carbohydrate need not be considered since carbohydrates do not stain

Figure 10. Electropherogram component patterns (SLS gel system) of membrane samples (CBB stained).
A. Non-stripped whole membrane (45 ug)
B. Non-stripped whole membrane (30 ug)
C. Stripping material (80 ul)



10

with CBB. The percent of phospholipid also need not be considered in comparing electropherogram stain intensities since lipids were found to migrate separately (near the gel front with protein species 43) away from all or most of the other membrane proteins. Therefore, using the 15 ug figure, it can be calculated that the amount of protein removed from 20 mg of whole membrane by stripping is probably a little less than 188 ug or 1.88 percent of the total protein present.

The protein removed by stripping (188 ug) was also calculated to be approximately 2.17 percent of the total amount of material stripped from the cell membrane. This figure was obtained by using 2,860 ug as the amount of carbohydrate present in strippings from 20 mg of whole membrane rather than 4,400 ug (based on 22% as carbohydrate) since further experimentation in our laboratory has revealed that 65 percent of the carbohydrate material present in the membrane of M. lysodeikticus is removed by the stripping procedure (King, unpublished data).

In summary, experiments a through f reveal that, regardless of additional type treatments employed, increased resolution of membrane proteins could not be effected. Therefore, it can be concluded that a valid pattern, and therefore examination, of the number of proteins present in the membrane of M. lysodeikticus can be obtained using SLS gel electrophoresis. In addition, the production of artifacts in this system, as far as can be determined, ap-

pears to be nominal or non-existent.

Effects of Varying Aggregation Parameters (Temperature, pH and Magnesium concentration)

Butler et al. (1967) reported that temperature, pH and magnesium ion concentration of the dialysis buffer affects the degree of aggregation. Optimum dialysis conditions (based on amount of aggregate formed) were reported to be a temperature of 37° C, 0.01 M magnesium ion concentration and a near neutral pH (7.4).

Rice (1969) was able to reaggregate all the disaggregated envelope components from Erwinia species into ordered sheets and found that the pH and magnesium ion concentration of the dialysis buffer affects the degree as well as the component species entering into aggregation.

Using disaggregated membrane components from various species of Mycoplasma, Razin, Ne'eman and Ohad (1969) reported that magnesium ion concentration during dialysis influences the degree of aggregation, the protein-to-lipid ratio present in the aggregate, and also the component species entering into aggregation.

Because the work of Rice in our laboratory indicated that both pH and magnesium ion concentration influence aggregation of the envelope components from Erwinia species, I examined the effect of these and other parameters on aggregation using membrane components from M. lysodeikticus.

Effect of Temperature on Aggregation

Aggregation experiments were performed (at pH 7.4 in the presence of 0.01 M magnesium ion concentration) at various temperatures (25°, 37°, 67° and 90° C) to test heat stability of the membrane components. Although "aggregation" seems to occur at all temperatures tested (Figures 11, 12, 13 and 14), the temperature of 25° C was selected for further aggregation studies since rather large and ordered membranous sheet formation occurs at this temperature within a time period of twenty-four hours. Ordered sheet formation also occurs at 37° C; however, the sheets are smaller in size compared to those formed at 25° C. At temperatures above 37° C, aggregation was observed to occur rather quickly, and dense, white pellets were obtained. Based on their appearance using the electron microscope, I have concluded that these aggregates are probably un-natural or disordered.

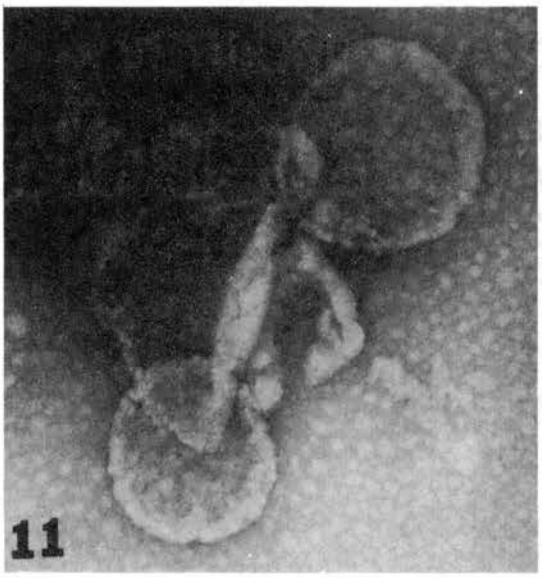
In summary, it appears that temperatures above 37° C do not cause sufficient denaturation to keep disaggregated membrane components from coming together. Higher temperatures do, however, denature sufficiently to disturb the appearance and ordered formation of the final product. Although only limited microscopic data are presented, the aggregation that occurs at higher temperatures (67° and 90° C) appears to be the result of disordered aggregation, i.e., aggregation by molecules which are denatured to various

Figure 11. Aggregates formed at 25° C (at pH 7.4 in the presence of 0.01 M Mg^{++}). Negatively stained with uranyl acetate (86,000X).

Figure 12. Aggregates formed at 37° C (at pH 7.4 in the presence of 0.01 M Mg^{++}). Negatively stained with uranyl acetate (86,000X).

Figure 13. Aggregates formed at 67° C (at pH 7.4 in the presence of 0.01 M Mg^{++}). Negatively stained with uranyl acetate (86,000X).

Figure 14. Aggregates formed at 90° C (at pH 7.4 in the presence of 0.01 M Mg^{++}). Negatively stained with uranyl acetate (86,000X).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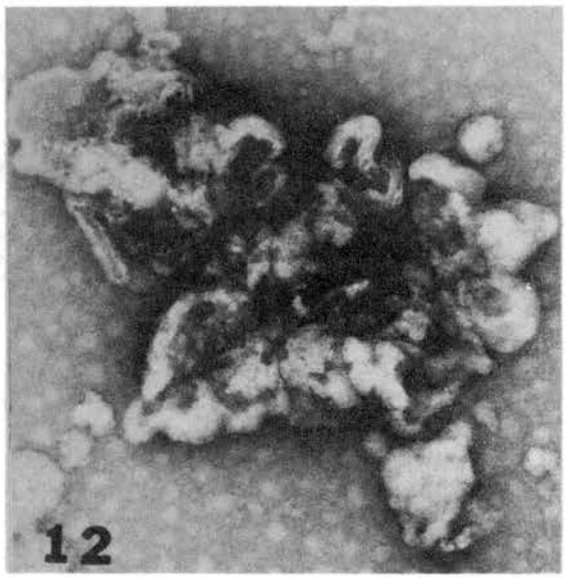
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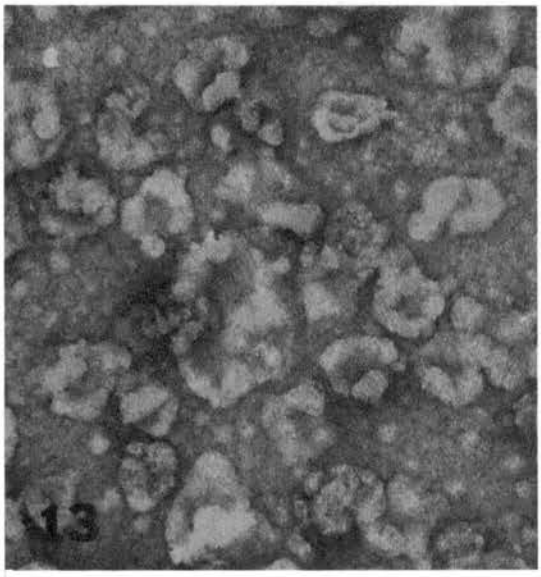
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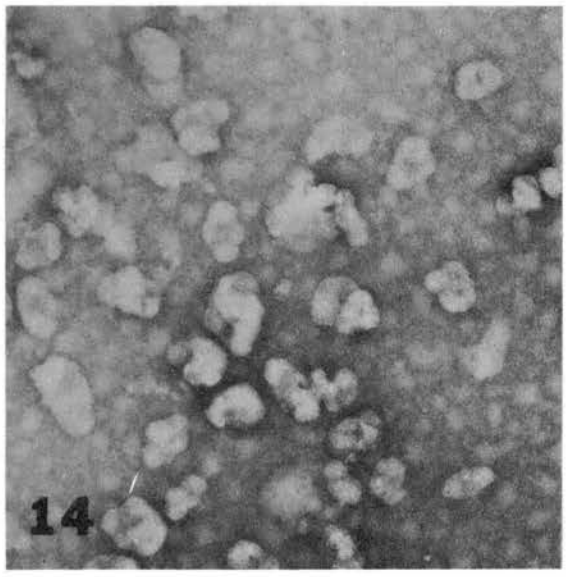
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degrees.

Effects of pH and Magnesium Concentration on Aggregation

To determine the effect(s) of pH and magnesium ion concentration on aggregation, disaggregated membrane components were reaggregated in 0.0025 M Tris buffers with pH adjustments to 4.0, 7.4 and 9.0 in the presence of either 0.01 or 0.001 M magnesium ion concentration. Aggregation experiments were always performed at 25° C for twenty-four hours unless otherwise specified. In addition, a buffer (B-buffer without B-mercaptoethanol present) was tested containing 0.0025 M Tris, 0.156 M sodium chloride and 0.01 M magnesium ions (Butler et al., 1967) with pH adjustments to 7.4 and 9.0 to determine the effect of sodium chloride on aggregation.

Data presented in Table III indicate that both pH and magnesium concentration significantly affect the degree of aggregation. It appears that there is an increase in amount of aggregation as the hydrogen ion concentration is increased. Further, it can be seen that the magnesium ion concentration does not greatly affect the degree of aggregation under acidic conditions (pH 4.0). It does appear, however, to be exerting an effect under basic conditions (either pH 7.4 or 9.0) in all buffers, with the amount of aggregate formed in the presence of 0.01 M magnesium ions approximately double that formed in 0.001 M magnesium ions. The effect of sodium chloride appears to be

TABLE III

EFFECT OF MAGNESIUM CONCENTRATION AND pH ON REAGGREGATION OF MEMBRANE COMPONENTS FROM *M. LYSODEIKTICUS*

pH of Buffer* and Concentration of Mg^{++} ions	Dry Weight of Aggregates (mg)**	O.D. (440 nm) of Supernatant After Aggregation	Percent Aggregation***
pH 4.0, 0.01 M	10.2	0.04	120
pH 4.0, 0.001 M	9.0	0.05	106
pH 7.4, 0.01 M	8.5	0.50	100
pH 7.4, 0.001 M	4.1	0.09	48
pH 7.4, *B*-buffer	8.2	0.05	96
pH 9.0, 0.01 M	8.0	0.05	94
pH 9.0, 0.001 M	4.0	0.10	47
pH 9.0, *B*-buffer	7.8	0.05	92

*Tris buffer (0.0025 M).

**Lyophilized to dryness prior to weighing.

***Amount of Aggregate formed at pH 7.4 in the presence of 0.01 M Mg^{++} was set at 100 percent.

nominal.

These dry weight data indicate that optimum aggregation occurs under acidic conditions in the presence of either 0.01 or 0.001 M magnesium ions. Examination using the electron microscope, however, revealed that ordered membranous sheets were not formed; instead, only small, vesicular-like, dense aggregates were present (Figures 15 and 16). Thus, it appears that membrane components are denatured in an acid pH. Contrary to this, it was found that aggregates formed under basic conditions are not denatured. Pictorial data reveal that aggregates of the ordered sheet type are formed at either pH 7.4 or 9.0 in the presence of either 0.01 or 0.001 M magnesium ions (Figures 17, 18, 19 and 20). The aggregates formed in 0.001 M magnesium, however, are smaller in size than those formed in 0.01 M magnesium. Thus, data obtained using the electron microscope (morphology of aggregated product) allow the conclusion that optimum conditions for aggregation of membrane components may be defined as pH 7.4 or 9.0 in the presence of 0.01 M magnesium ions.

To determine if the unusual sheet formation that results at pH 4.0 was due to denaturation of the membrane components at this relatively low pH, the aggregates were collected, disaggregated as usual, and then placed under dialysis conditions at pH 9.0 in the presence of 0.01 M magnesium ions for aggregation. The picture shown in Figure 21 reveals that ordered sheet formation will occur us-

Figure 15. Vesicular-like membrane aggregates formed at pH 4.0 in the presence of 0.01 M Mg^{++}. Negatively stained with uranyl acetate (120,000X).

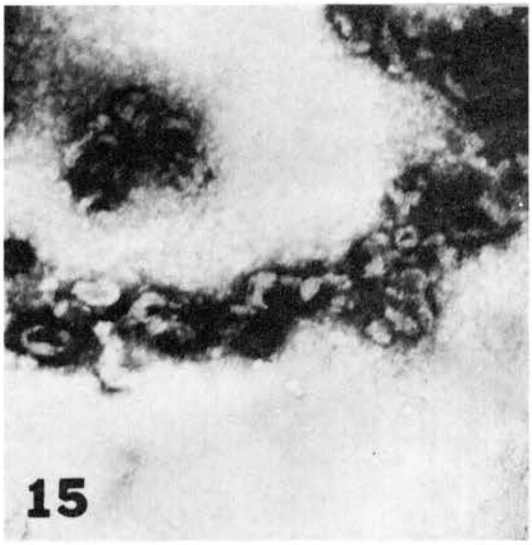
Figure 16. Vesicular-like membrane aggregates formed at pH 4.0 in the presence of 0.001 M Mg^{++}. Negatively stained with uranyl acetate (120,000X).

Figure 17. Membranous sheets formed at pH 7.4 in the presence of 0.01 M Mg^{++}. Negatively stained with uranyl acetate (60,000X).

Figure 18. Membranous sheets formed at pH 7.4 in the presence of 0.001 M Mg^{++}. Negatively stained with uranyl acetate (120,000X).

Figure 19. Membranous sheets formed at pH 9.0 in the presence of 0.01 M Mg^{++}. Negatively stained with uranyl acetate (60,000X).

Figure 20. Membranous sheets formed at pH 9.0 in the presence of 0.001 M Mg^{++}. Negatively stained with uranyl acetate (120,000X).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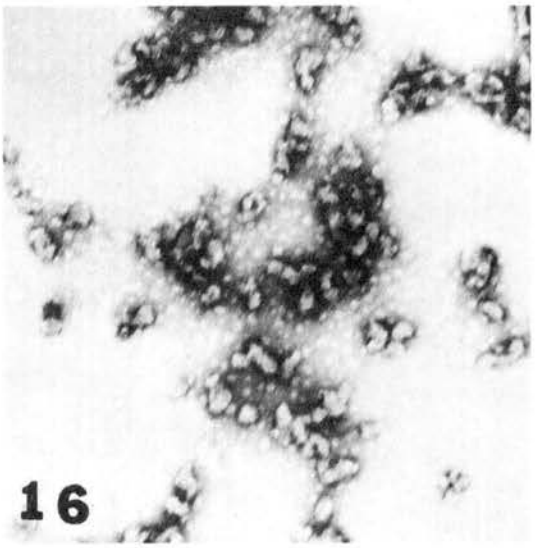
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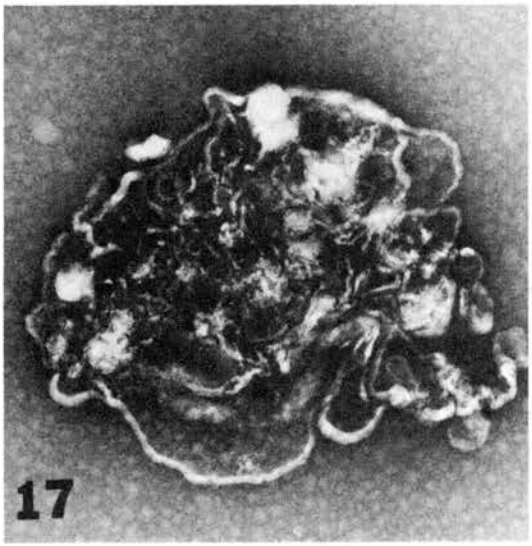
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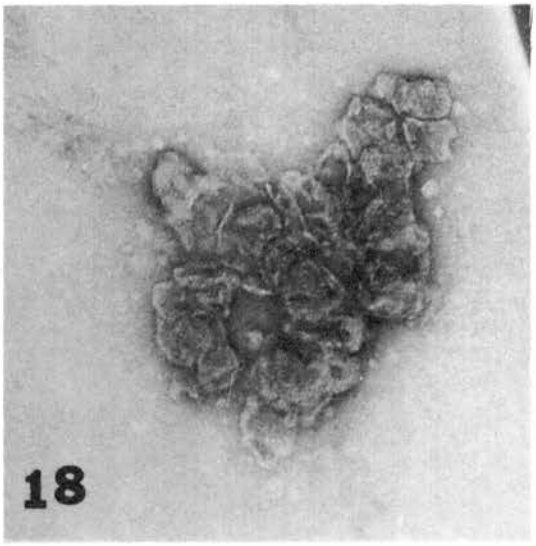
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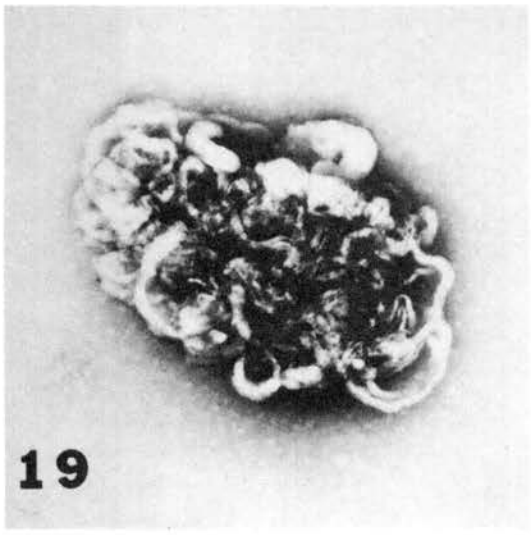
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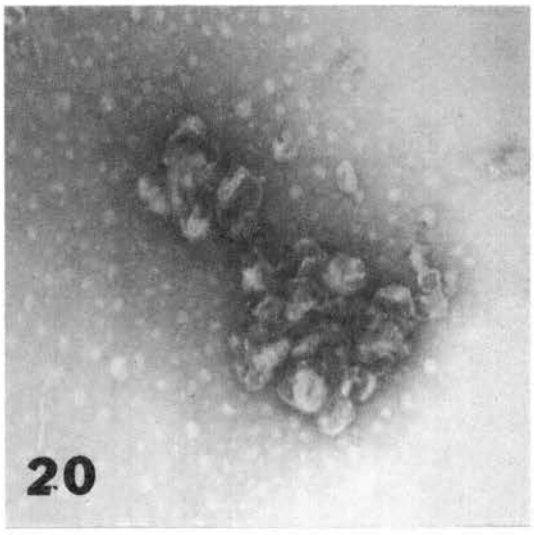

20

ing components from these vesicular-type aggregates.

Although extensive studies have not been performed, I feel that most of the insoluble material that forms during dialysis at pH 4.0 represents a type or form of associated membrane material rather than simply a precipitation. Two significant factors can be mentioned relative to these associated components. The association occurs quickly (within five to twenty minutes) after the dialysis bag containing the disaggregated membrane components is put at pH 4.0. This could indicate a simple precipitation; however, a second type of observation is pertinent. The pellets of aggregates formed under acidic conditions pack tightly after centrifugation, are opaque and yellow-white in color. When the pellet is resuspended at pH 9.0 and recentrifuged, the pellet now obtained is neither tightly packed nor opaque; instead, it spreads over a large area of the bottom of the centrifuge tube and is transparent. If this pellet is, in turn, resuspended at pH 4.0 and again centrifuged, the tightly packed, opaque and yellow-white pellet originally present is again obtained. This alternate shrinking and swelling (but not solubilization) indicates that protein molecules are involved, and their amphoteric nature is being expressed at the high and low pH values. These observations further indicate that some type of association between the protein molecules is occurring because they could not be sedimented (54,500 x g for thirty minutes) if they existed as individual entities. This alternate shrink-

ing and swelling phenomenon also occurs with whole membrane or aggregates formed at pH 7.4 or 9.0, and is similar to that reported for whole bacteria adjusted to high and low pH values after treatment with lysozyme (Grula and Hartsell, 1957).

Electrophoretic analysis revealed that the aggregates formed under acid conditions in the two concentrations of magnesium ions (either 0.01 or 0.001 M) are similar qualitatively, but differ quantitatively (Figure 22A,B). The quantitative differences were most significant in the high MW range (above 60,000 daltons), with component species 15, 16 and 17 almost completely absent in aggregates formed in the presence of 0.001 M magnesium. The major component species present in the aggregate formed in the presence of the higher magnesium ion concentration (0.01 M) appear to be 15, 17, 20, 23, 28, 30, 31, 36, 37 and 40, whereas species 20, 23, 28, 30, 31, 36, 37 and 40 appear to be the major species present in the aggregates formed in the presence of the lower magnesium ion concentration (0.001 M). In addition, it was noted that larger amounts (260 ug) of the aggregate formed in the lower magnesium concentration were required for good visualization of proteins after electrophoretic analysis as compared to the aggregate formed in the higher magnesium concentration (180 ug).

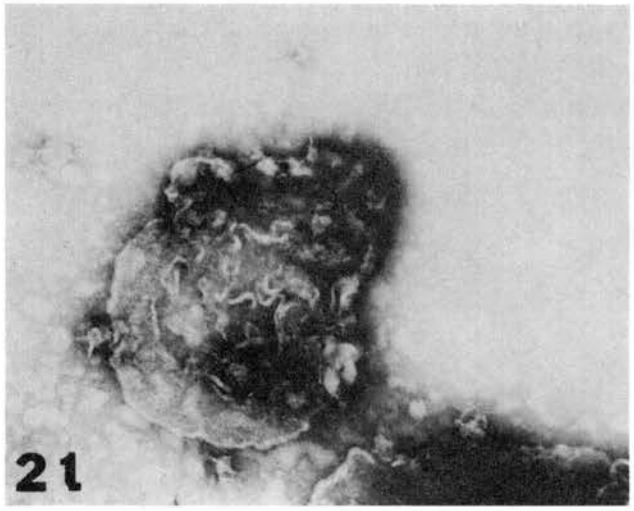
To more accurately determine which component species were not entering the aggregates formed at pH 4.0 in the presence of 0.01 M magnesium, the supernatant from these

Figure 21. Ordered membranous sheets formed at pH 9.0 in the presence of 0.01 M Mg^{++} made using disaggregated membrane components previously aggregated at pH 4.0 in the presence of 0.01 M Mg^{++}. Negatively stained with uranyl acetate (86,000X).

Figure 22. Electropherogram component patterns obtained from membrane aggregates formed under acidic (pH 4.0) conditions (CBB stained).
A. in the presence of 0.01 M Mg^{++} (180 ug)
B. in the presence of 0.001 M Mg^{++} (260 ug)

Figure 23. Membrane material formed after dialyzing (at pH 7.4 in the presence of 0.01 M Mg^{++}) the supernatant from aggregates formed at pH 4.0 in the presence of 0.01 M Mg^{++}. Negatively stained with uranyl acetate (120,000X).

Figure 24. SLS electrophoretic gel columns containing resolved membrane components (CBB stained).
A. Isolated whole membrane (120 ug)
B. Material pictured in Figure 23 (100 ug)



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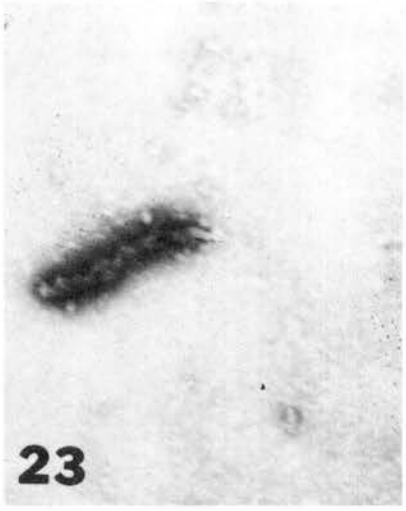

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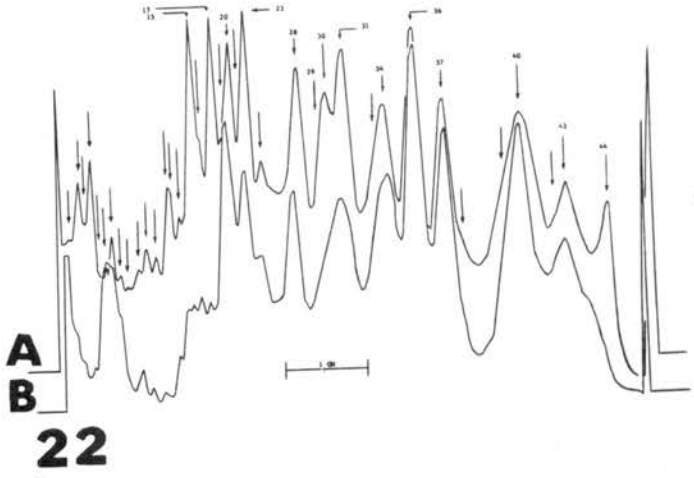

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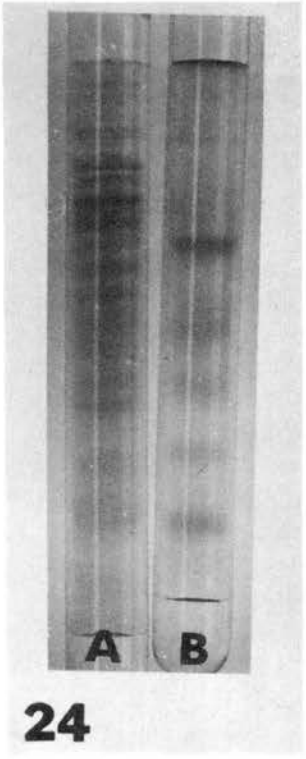


24

was dialyzed for aggregation at pH 7.4 in the presence of 0.01 M magnesium ions. An aggregate consisting only of small pieces (no ordered sheet or vesicular-type structures) was obtained (Figure 23). Electrophoretic analysis of this material revealed that several component species (about eight) were present; however, only species 26 was present in large amount (Figure 24). This information indicates that the membrane component species 26 is not essential for ordered membranous sheet formation during aggregation even though it may have the ability to aggregate with other components into some types of unordered structures.

As previously stated, membrane aggregates formed under basic conditions (either pH 7.4 or 9.0) in the presence of either 0.01 or 0.001 M magnesium ion concentration appear to be different as indicated by electron microscopic examination. To ascertain if these membrane aggregates differ in component composition, SLS gel electrophoretic analyses were performed.

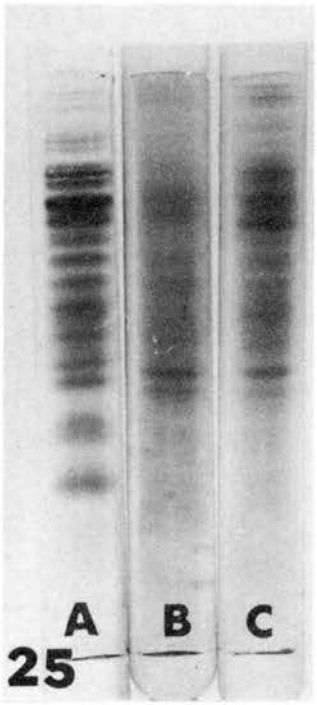
As shown in Figures 25A,B,C and 26A,B,C, membrane aggregates, formed under the basic conditions described above, exhibit membrane component patterns which differ from the pattern obtained from the whole membrane of M. lysodeikticus. In general, most of the differences are quantitative. The few qualitative differences which occur appear to be in the higher MW species (above 55,000 daltons). More specifically, the component pattern obtained from membrane aggregates formed at pH 7.4 in the presence of 0.01 M magnes-

Figure 25. SLS electrophoretic gel columns containing resolved membrane components (CBB stained).
A. Isolated whole membrane (120 ug)
B. Membrane aggregates formed at pH 7.4 in the presence of 0.01 M Mg^{++} (150 ug)
C. Membrane aggregates formed at pH 7.4 in the presence of 0.001 M Mg^{++} (210 ug)

Figure 26. SLS electrophoretic gel columns containing resolved membrane components (CBB stained).
A. Isolated whole membrane (120 ug)
B. Membrane aggregates formed at pH 9.0 in the presence of 0.01 M Mg^{++} (120 ug)
C. Membrane aggregates formed at pH 9.0 in the presence of 0.001 M Mg^{++} (210 ug)

Figure 27. Electropherogram component patterns obtained from membrane aggregates formed at pH 7.4 (CBB stained)
A. in the presence of 0.01 M Mg^{++} (150 ug)
B. in the presence of 0.001 M Mg^{++} (210 ug)

Figure 28. Electropherogram component patterns obtained from membrane aggregates formed at pH 9.0 (CBB stained)
A. in the presence of 0.01 M Mg^{++} (120 ug)
B. in the presence of 0.001 M Mg^{++} (210 ug)



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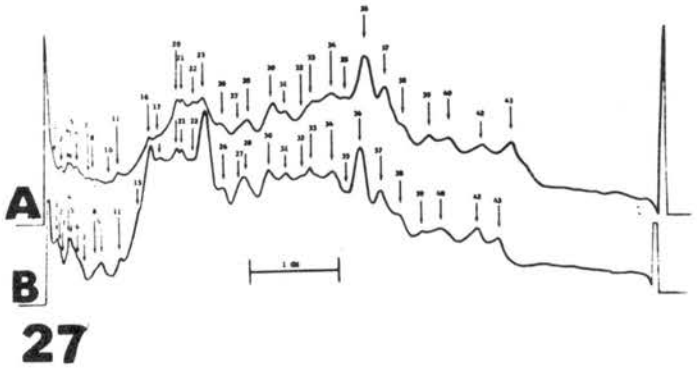

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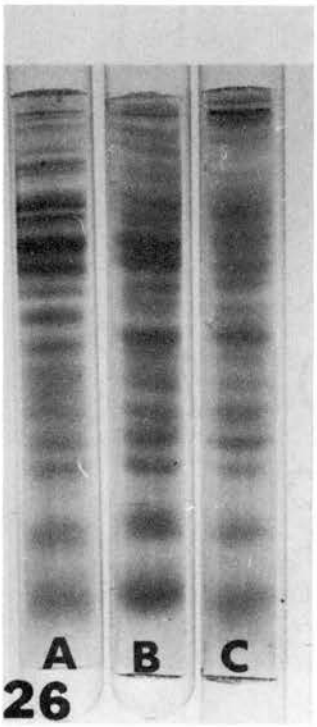

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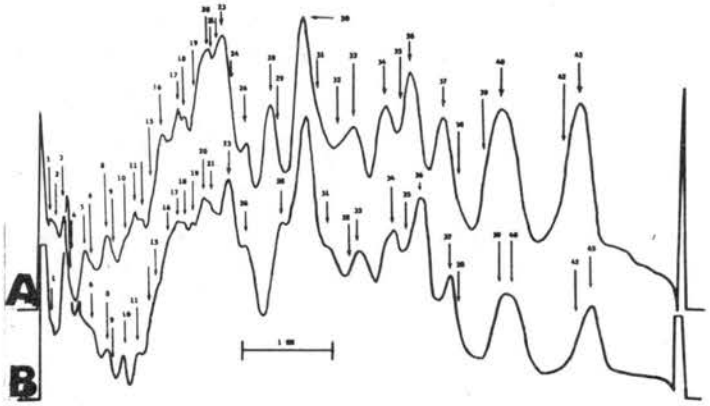


28

ium ion concentration reveals that most component species enter into aggregation under these conditions (Figures 25B and 27A). Based on stain intensity, they do not appear to be present in the aggregates in the same proportion as they are in the whole membrane pattern. Even though the picture of the gel column is not of good quality (Figure 25B), it can be seen by directly observing the stained column that the major species in the aggregate possess MW in the range from 20,000 to 65,000 daltons (20, 23, 28, 30, 36 and 37). Further, few species with MW greater than 65,000 daltons (exceptions are species 2 through 6, 11 and 16) appear to be present in amounts sufficient to be detected by densitometer scanning (Figure 27A).

The electropherogram component pattern obtained from membrane aggregates formed at pH 7.4 but in the presence of 0.001 M magnesium reveals that most species also appear to be entering into aggregation under these conditions (Figures 25C and 27B). Further, the major species present in the aggregate also appear to be in the MW range from 20,000 to 65,000 daltons, i.e., species 16, 20, 23, 28, 30, 36 and 37. Consequently, this pattern appears to be similar to that obtained from the aggregate formed at the higher magnesium concentration (0.01 M). Unlike aggregation in the presence of 0.01 M magnesium, however, the lower magnesium concentration (0.001 M) appears to be more favorable for species with MW greater than 65,000 daltons to enter into aggregation. Perhaps the most significant difference

between the two aggregates (as determined by pattern stain intensities) is that component species 16 and 23 appear to enter into aggregation to a greater extent in the presence of the lower magnesium concentration.

The similarity in electropherogram component patterns obtained from aggregates formed at pH 7.4 in the presence of either 0.01 or 0.001 M magnesium concentration indicates that the structures of the aggregates formed in the lower magnesium concentration are of the ordered sheet type, which are restricted in size only because of the limited amount of magnesium ions available during aggregation. Magnesium appears to be limiting at the concentration of 0.001 M since an increase in sheet size does not occur with continued dialysis up to seventy-two hours. It was noted, however, that significantly larger amounts (approximately 40% more) of aggregates formed in the lower concentration of magnesium are required to give an electropherogram pattern of equal stain intensity to that obtained from an aggregate formed in the higher magnesium concentration. This information indicates that the component (protein, phospholipid and carbohydrate) ratios may be different in these membrane aggregates.

Electrophoretic analysis of membrane aggregates formed at pH 9.0 in the presence of either 0.01 or 0.001 M magnesium concentration also revealed them to possess similar gel and electropherogram patterns (Figures 26A,B,C and 28A,B). As in the aggregates formed at pH 7.4, the major species

entering into aggregation appear to be those with MW less than 65,000 daltons. As determined by pattern intensity, species 20, 23, 28, 30, 36, 37, 40 and 43 appear to be the major species present in the aggregates formed in the presence of the higher magnesium concentration (0.01 M), whereas species 16, 20, 23, 28, 30, 36, 37, 40 and 43 appear to be the major species present in the aggregate formed in the lower magnesium concentration (0.001 M). Again, it was noted that significantly larger amounts (approximately 40% more) of aggregates formed in the lower concentration of magnesium are required to give an electropherogram pattern of equal stain intensity to that obtained from the aggregates formed in the higher magnesium concentration.

In addition to these data, it appears that aggregation at pH 9.0 results in patterns which are more similar to that obtained from whole membrane than aggregation at pH 7.4 (compare Figures 25A,B,C; 26A,B,C; 27A,B; and 28A,B). This information suggests that optimal aggregation occurs at the higher pH (9.0).

Overall, my data indicate that both pH and magnesium ion concentration influence which component species will enter into aggregation. As an example of pH effects, component species 30 appears to be the major one entering into aggregation at pH 9.0, whereas number 23 and 36 appear to be the major ones entering at pH 7.4. The major species present in the membrane aggregates formed under the conditions tested are shown in Table IV. No one species appears

TABLE IV

MAJOR COMPONENT SPECIES PRESENT IN AGGREGATES FORMED UNDER CONDITIONS TESTED*

Membrane Material	Species Number**
Aggregates formed at pH 4.0 in the presence of 0.01 M Mg^{++}	15 17 20 23 28 30 31 36 37 40
Aggregates formed at pH 4.0 in the presence of 0.001 M Mg^{++}	20 23 28 30 31 36 37 40
Aggregates formed at pH 7.4 in the presence of 0.01 M Mg^{++}	20 23 28 30 36 37
Aggregates formed at pH 7.4 in the presence of 0.001 M Mg^{++}	16 20 23 28 30 33 36 37
Aggregates formed at pH 9.0 in the presence of 0.01 M Mg^{++}	20 23 28 30 36 37 40 43
Aggregates formed at pH 9.0 in the presence of 0.001 M Mg^{++}	16 20 23 28 30 33 36 37 40 43

*As determined by electropherogram pattern stain intensities.

**All species are present in all membrane aggregates. To determine gel column position and appearance as well as estimated MW, see Figure 4A,B.

to constitute the majority of protein present in any of the aggregates; however, it can be seen that several (20, 23, 28, 30, 36 and 37) are always present as major components in all the aggregates.

Because aggregates formed in the presence of low levels of magnesium (0.001 M) always showed less protein (based on gel column staining) per unit weight of aggregates, radioisotope-labeled membrane components were utilized in aggregation experiments to determine which membrane component (protein, phospholipid or carbohydrate) is most responsive to the level of magnesium ions during aggregation. In the first experiment, different batches of the organism were grown in the presence of different compounds (acetate-2-^{14}C, L-aspartate-U-^{14}C and L-glutamate-U-^{14}C) to label the cell membrane. Membranes were obtained, disaggregated and reaggregated as usual and the aggregates counted.

The results shown in Table V reveal that approximately 60 percent of the counts (membrane components) enter into aggregation in the presence of 0.01 M magnesium, whereas only about 20 percent enter in the presence of 0.001 M magnesium. These data clearly demonstrate that about three times more aggregation occurs in the presence of 0.01 M magnesium than in the presence of 0.001 M magnesium ion concentration. Although obtained in a different way, our conclusion is in agreement with Butler et al. (1967), Rice (1969) and Razin et al. (1969).

In a further experiment, labeled membrane aggregates

TABLE V

EFFECT OF MAGNESIUM CONCENTRATION ON AGGREGATION

Conditions and Type of Sample*	Counts Recovered**			Percent of Count Appearing in the Aggregates		
	L-aspartate	Acetate	L-glutamate	L-aspartate	Acetate	L-glutamate
Aggregates formed at pH 7.4 in the presence of 0.01 M Mg^{++}	6,325	22,609	22,840	61.6	61.2	56.0
Supernatant from above	3,939	14,332	18,020			
Aggregates formed at pH 7.4 in the presence of 0.001 M Mg^{++}	1,920	7,889	9,620	16.6	19.5	21.2
Supernatant from above	9,621	32,568	35,690			

*Aggregated twenty-four hours at 25° C in Tris buffer (0.0025 M).

**Whole membranes had been isolated from cells grown in the presence of Acetate-2-^{14}C (0.002 uC/ml), L-aspartate-U-^{14}C (0.004 uC/ml) or L-glutamate-U-^{14}C (0.0025 uC/ml).

(separately prepared using L-aspartate-U-^{14}C, L-glutamate-U-^{14}C and glycerol-1-^{14}C as previously described) were stripped to ascertain if the concentration of magnesium also effects the protein-to-lipid ratio present in the aggregates. Data are given in Table VI.

In the presence of 0.01 M magnesium, the aggregates have a protein-to-lipid ratio of about 2.7 to 1, whereas in those aggregates formed in the presence of 0.001 M magnesium, a ratio of about 1.5 to 1 is evident. These data aid us in understanding why greater amounts of the aggregates formed at lower magnesium ion concentrations are necessary for SLS gel electrophoretic analysis of proteins. Since the protein-to-lipid ratio is about 1.7 to 1 in whole membranes, these data support the concept that, regardless of the conditions used, membranous sheets formed during aggregation are quantitatively different from whole membranes.

Overall, it appears that aggregation of proteins is more dependent on magnesium ion concentration than lipids. This conclusion was also reached by Razin et al. (1969). It may occur because negatively charged phosphate groups on phospholipids can more readily attract and hold magnesium ions. Such priority trapping of magnesium ions would be of significance for membrane fabrication if a lipid bilayer must first be formed onto which protein is then added.

In summary, it was found that both pH and magnesium ion concentration during dialysis significantly influence reaggregation of disaggregated membrane components of M.

TABLE VI

EFFECT OF MAGNESIUM CONCENTRATION ON PROTEIN-LIPID RATIOS IN MEMBRANE AGGREGATES*

Conditions and Type of Sample**	Total Counts Present***			Protein to Lipid Ratio****		
	L-aspartate	Glycerol	L-glutamate	L-aspartate	Glycerol	L-glutamate
Whole Membrane			29,663			1.67
Strippings from above			17,750			
Aggregates formed at pH 7.4 with 0.01 M Mg^{++}	2,613	5,162	7,826	2.88	2.55	2.67
Strippings from above	906	2,027	2,926			
Aggregates formed at pH 7.4 with 0.001 M Mg^{++}		800	3,442		1.60	1.44
Strippings from above		501	2,391			

*Whole membranes had been isolated from cells grown in the presence of L-aspartate-U-^{14}C (0.004 uC/ml), L-glutamate-U-^{14}C (0.005 uC/ml in the whole membrane sample and 0.0025 uC/ml in the other samples) or Glycerol-1-^{14}C (0.005 uC/ml).

**Aggregated for twenty-four hours at 25° C in Tris buffer (0.0025 M).

***Total counts present are adjusted for amount protein present in stripping materials (2.17%), quenching of the stripping materials by acetone reagent (X1.35) and for percent carbohydrate composition (whole membrane, 13%; aggregates formed in 0.01 M Mg^{++}, 10%; and aggregates formed in 0.001 M Mg^{++}, 6%).

****Lipid is one in all cases.

lysodeikticus. Aggregation under acidic conditions (pH 4.0) causes an unordered-type association of membrane components, and magnesium concentration has little effect on the final product as regards amount formed, protein species present and morphology of the aggregates. Aggregation under basic conditions (pH 7.4 or 9.0) favors the formation of ordered membranous sheet structures. Concentration of magnesium ions present during dialysis determines the amount of aggregate obtained, the protein-to-lipid ratios within the aggregates, the size of the membrane sheets and, to a lesser extent, degree of entry of certain protein species into aggregation. The higher pH (9.0) appears to favor a more natural type of membrane product because of similarity in protein composition to isolated whole membrane. My data are consistent with the conclusion that, regardless of the composition of the dialysis buffer during aggregation, other than morphological appearance, none of the fabricated membranous sheets are the same as native whole membrane.

Phospholipid and Carbohydrate Studies of Membrane Materials

Since the membrane of M. lysodeikticus is composed of protein, phospholipid and carbohydrate (Gilby et al., 1958), it seems feasible that its structure should result from various types of interactions between these components.

The occurrence and importance of protein-to-protein

interactions (van der Waals forces) in the membrane of M. lysodeikticus has been emphasized by Grula et al. (1967). As a result of stripping experiments, these investigators suggested that most phospholipids and carotenoids appear to be present as superstructures on the top and bottom of structural protein; their function is to protect structural protein forming the basal continuum and anchor other proteins having enzymatic activity. The following experiments were done to aid us in understanding further the role(s) of phospholipids and carbohydrates in membrane structures.

Phospholipid Studies

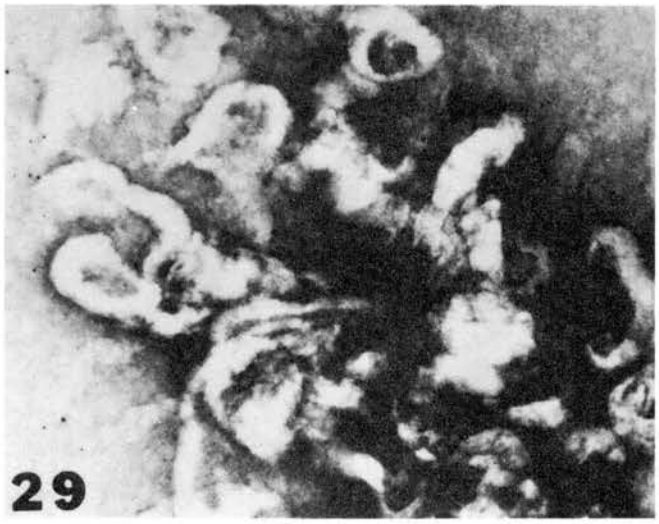
To determine whether or not phospholipids and carotenoids exert control and somehow dictate which proteins enter into the aggregated state, protein patterns of stripped aggregates were obtained.

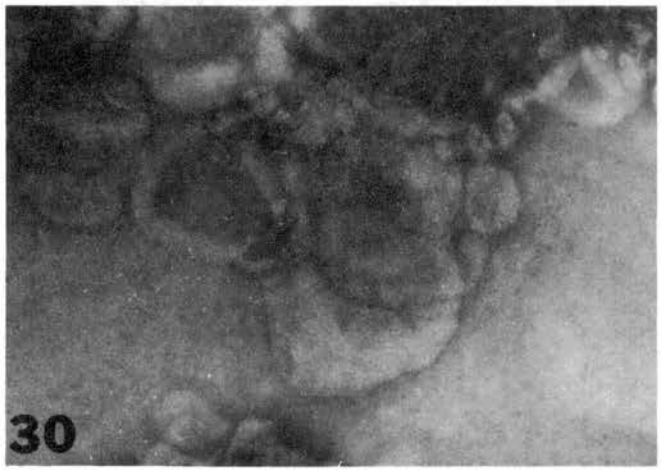
For these experiments, whole membranes were stripped, washed, disaggregated, SLS removed as usual and the membrane components reaggregated under conditions optimal for ordered sheet formation (either pH 7.4 or 9.0 in the presence of 0.01 M magnesium ion concentration). Observation of such aggregates using the electron microscope revealed that membranous sheets do indeed form (Figures 29 and 30).

To determine the effect of stripping on the total amount of aggregation possible, membranes labeled during growth using acetate-2-^{14}C were stripped and the amount of

Figure 29. Membranous sheets formed from stripped membranes at pH 7.4 in the presence of 0.01 M Mg^{++}. Negatively stained with uranyl acetate (120,000X).

Figure 30. Membranous sheets formed from stripped membranes at pH 9.0 in the presence of 0.01 M Mg^{++}. Negatively stained with uranyl acetate (120,000X).


29


30

aggregation obtained was compared to non-stripped controls. The results shown in Table VII reveal that the degree of aggregation is greatly affected by stripping, i.e., there is a 73 percent decrease in amount of aggregate formed from stripped membranes when the components are aggregated in the presence of 0.01 M magnesium ions and a 93 percent decrease when aggregation is accomplished in 0.001 M magnesium. The fact that the percent decreases in aggregation under both conditions are large could indicate that few, but similar, component species are entering into aggregation.

To actually determine if stripping caused exclusion of some proteins during aggregation, aggregates formed from stripped materials were analyzed and found to be almost identical (Figures 31A,B and 32B,C). Although component species 30 appears to be present in greater quantity in the aggregate formed in the higher concentration of magnesium (0.01 M), the major species present in both types of membrane aggregates are 20, 21, 23, 28, 30, 36, 40 and 43. It can also be seen that some material 'stacked' on top of the columns during these analyses. This indicates that some denaturation may have occurred as a result of stripping, and thus, the patterns may not be a true representation of all proteins present in the membranous sheets. The primary difference between these patterns and the patterns obtained from membrane aggregates formed from non-stripped membranes at pH 7.4 in the presence of either 0.01

TABLE VII

EFFECT OF STRIPPING ON AGGREGATING ABILITY*

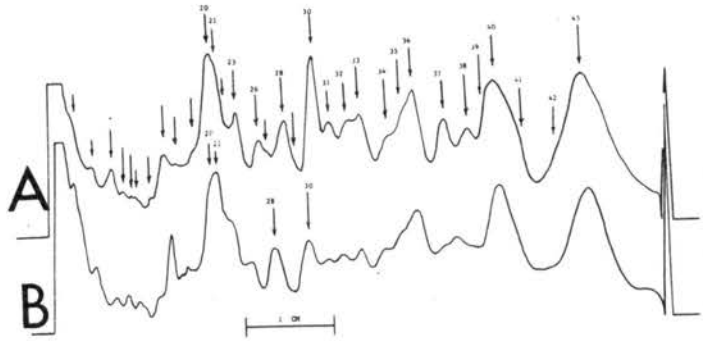
Conditions and Type of Sample**	Counts Recovered	Percent of Counts Entering into Aggregation	Percent Decrease in Aggregation Due to Stripping
Non-stripped Membranes			
Aggregates formed at pH 7.4 with 0.01 M Mg^{++}	22,608	61.2	
Supernatant from above	14,332		
Aggregates formed at pH 7.4 with 0.001 M Mg^{++}	7,889	19.5	
Supernatant from above	32,568		
Stripped Membranes			
Aggregates formed at pH 7.4 with 0.01 M Mg^{++}	2,035	16.4	73.0
Supernatant from above	10,392		
Aggregates formed at pH 7.4 with 0.001 M Mg^{++}	235	1.75	93.0
Supernatant from above	13,182		

*Membranes isolated from cells grown in the presence of acetate-2-^{14}C.

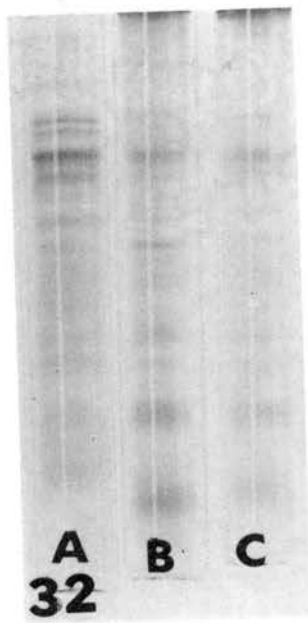
**Aggregated twenty-four hours at 25° C in Tris buffer (0.0025 M).

Figure 31. Electropherogram component patterns obtained from stripped membrane aggregates formed at pH 7.4 (CBB stained)
A. in the presence of 0.01 M Mg^{++} (120 ug membranes)
B. in the presence of 0.001 M Mg^{++} (150 ug membranes)

Figure 32. SLS electrophoretic gel columns containing resolved membrane components (CBB stained).
A. Isolated whole membrane (120 ug)
B. Material described in Figure 31A (120 ug)
C. Material described in Figure 31B (150 ug)



31



32

or 0.001 M magnesium ion concentration appears to be that component species 30, 40 and 43 are present in greater amount in stripped membrane aggregates.

Since the proteins present in the stripped membranous aggregates are not significantly different from control aggregates wherein phospholipids and carotenoids are present, these data lend support to the concept that phospholipids and carotenoids are present as superstructures, and their primary function derives from this type association. Because all proteins appear to be present in aggregates formed from stripped membrane materials, it is difficult to ascribe an anchoring function to phospholipids and carotenoids; however, because significantly less aggregation occurs using components from stripped membranes, a protective function (from denaturation or maintenance of the correct conformational state) for phospholipids and carotenoids remains a distinct possibility.

It has been reported that mammalian mitochondrial membrane "subunits", stripped of lipid materials, form random three-dimensional aggregates (disordered structures) and only after lipid has been reintroduced into the stripped units will ordered sheet formation occur (Green et al., 1967). Although a subunit type structure as originally envisioned does not appear to exist in the membrane of M. lysodeikticus (Grula and Savoy, 1971), I did experiments relative to the hypothesis of Green et al. (1967) by first stripping the cell membrane of M. lysodeikticus using ace-

tone reagent, solubilizing the stripped membrane in SLS then stripping the solubilized proteins again using the acetone reagent. When done in this manner, stripping causes precipitation of SLS-solubilized proteins and they can easily be collected by centrifugation. After sedimentation, the proteins were resuspended in 0.0025 M Tris buffer (pH 7.4), and detergent again added for re-solubilization. After removal of detergent by incubation overnight at 4° C as usual, aggregation of this type stripped protein was allowed to proceed for forty-eight hours. Data given in Table VIII reveal that aggregation of such stripped proteins will occur.

TABLE VIII

REAGGREGATION OF STRIPPED MEMBRANE COMPONENTS OF M. LYSODEIKTICUS*

Situation	Optical Density		
	440 nm	540 nm	660 nm
Before Dialysis	0.07	0.03	0.02
After Dialysis	0.35	0.20	0.13

*Components stripped by acetone reagent before and after detergent (SLS) solubilization.

Observation of these aggregates using the electron microscope revealed that small and relatively thick aggregates were indeed present (Figure 33). When the extracted phospholipids and carotenoids were added back into the di-

alysis bag prior to aggregation, some sheet formation occurred; however, the process appeared to be very slow and inefficient and the small aggregates were still the dominant structures present (Figure 34).

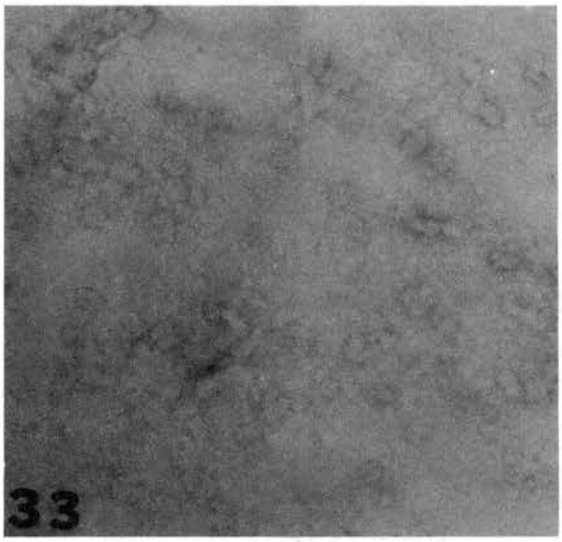
Overall, our data indicate that if stripping of proteins is accomplished while the membrane is intact, significant amounts of the resulting stripped proteins will, under appropriate conditions, aggregate into an ordered membranous structure. If, however, membrane proteins are first solubilized and then stripped, the resulting stripped proteins will not aggregate into ordered membranous sheets. Although denaturation of other portions of the protein molecules may occur, it is possible that the interface areas (van der Waals contact and bonding areas), when exposed directly to acetone, are the critical areas to undergo some type of conformational change or changes (denaturation). These interface areas may not be extensively denatured when the intact membrane is stripped because protein-to-protein associations protect them from the damaging effects of the stripping reagent. Because of these data, it appears that caution should be exercised before concluding that stripping has no effect on membrane proteins or appearance of the resulting product.

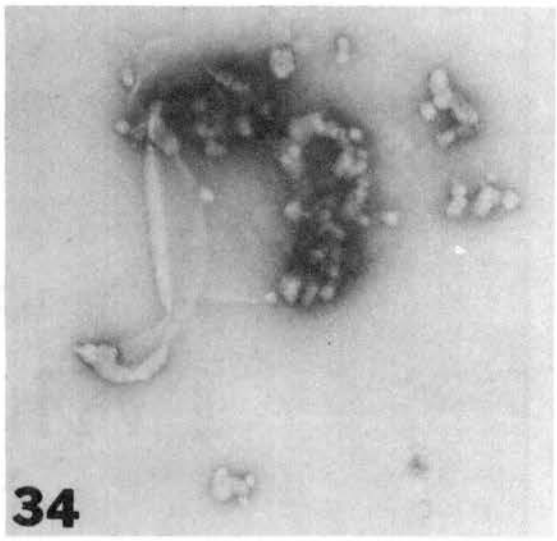
Some investigators are of the opinion that a lipid bilayer is the fundamental unit of membrane structure; Korn (1966), Stoeckenius (1966) and Lucy and Glauest (1967) have reported that layered, membranous structures (myelin fig-

Figure 33. Membrane aggregates formed from stripped solubilized membrane proteins at pH 7.4 in the presence of 0.01 M Mg^{++}. Negatively stained with uranyl acetate (43,000X).

Figure 34. Membranous sheets and small aggregates formed from stripped solubilized membrane proteins in the presence of added phospholipids and carotenoids. Conditions were given in Figure 33. Negatively stained with uranyl acetate (43,000X)

Figure 35. Phospholipids and carotenoids precipitated in the presence of 0.01 M Mg^{++} at pH 7.4. Negatively stained with uranyl acetate (86,000X).


33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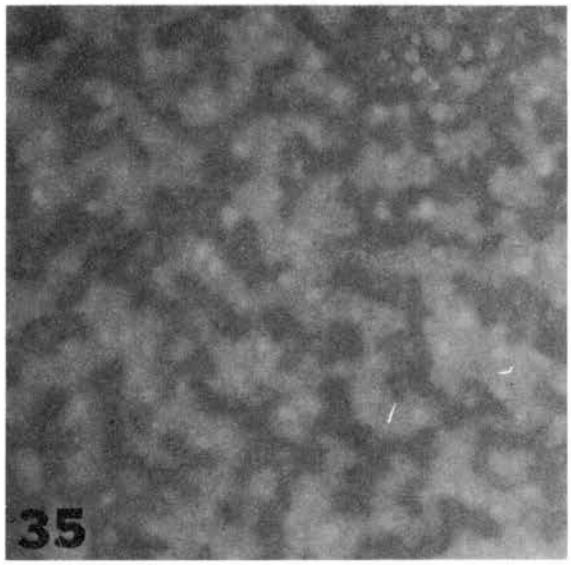

35

ures) could be obtained by mixing lipids with water. They have also pointed out that a variety of shapes can occur if cholesterol and saponin are also present.

To determine if phospholipids and carotenoids from M. lysodeikticus membranes can form some type of ordered membranous structure, stripping materials were, by themselves, dialyzed for aggregation (at pH 7.4 in the presence of 0.01 M magnesium ion concentration). Although several different extracts were tested, no membranous structures were ever fabricated (Figure 35).

In summary, it was found that membranous sheets are formed under optimal conditions of dialysis from M. lysodeikticus membrane materials which have been stripped of phospholipids and carotenoids prior to disaggregation. Although aggregation and sheet formation occur in the absence of lipids, it was observed that the degree of aggregation is greatly increased when lipids are present. Whether this occurs because of some denaturation of membrane proteins by the acetone stripping reagent or whether it indicates a need for lipids cannot yet be determined. It has been observed that phospholipids and carotenoids will not, by themselves, form any type of ordered sheet structure during extended dialysis.

Carbohydrate Studies

To further determine whether or not glycoproteins may be present in these membranes, aggregates (formed at pH 9.0

in the presence of either 0.01 or 0.001 M magnesium ion concentration) were stripped and analyzed two different ways: (1) anthrone test procedure for carbohydrates (Dr. Billy Hudson, personal communication) and (2) glycoprotein staining of proteins in SLS gel columns.

We assumed that if mannose exists as a homopolysaccharide in the membrane of M. lysodeikticus as described by MacFarlane (1964), it should not be disaggregated by SLS and should therefore be removed during the preparative centrifugation steps. Further, it was assumed that even if mannan molecules were not removed during centrifugation they would not enter into aggregation; instead, they would remain in the supernatant during sedimentation and washing of the aggregates.

The results shown in Table IX reveal that large amounts of carbohydrate were present in all aggregate samples tested even though most phospholipid and carotenoid materials had been removed by stripping prior to testing. The presence of carbohydrate materials in membrane aggregates established that carbohydrate components are entering into aggregation. Further, it is very likely that substantial amounts of carbohydrate are present as glycoproteins in the membrane of M. lysodeikticus since staining of gels, using the glycoprotein staining procedure, revealed that several glycoproteins are present: 11, 16, 20, 23, 26, 28 and 30. The major species (20) present in whole membranes is also the major one present in stripped membrane aggregates (com-

TABLE IX

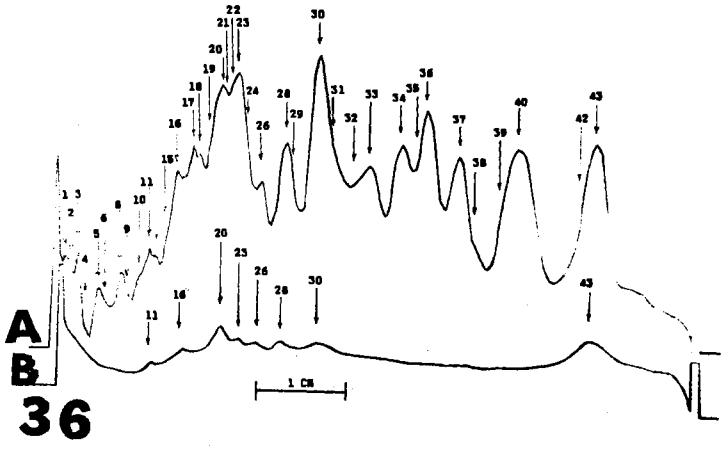
PRESENCE OF CARBOHYDRATE IN VARIOUS TYPES OF MEMBRANES

Membrane Sample	Total ug of Carbohydrate per mg Sample*	Percent Carbohydrate
Whole Membrane	65.0	6.5
Aggregates formed at pH 9.0 in the presence of 0.01 M Mg^{++}	50.0	5.0
Aggregates formed at pH 9.0 in the presence of 0.001 M Mg^{++}	30.0	3.0

*Estimations based on a glucose standard curve. Samples were stripped prior to analysis.

Figure 36. Electropherogram component patterns obtained from membrane aggregates formed at pH 9.0 in the presence of 0.01 M Mg^{++} (120 ug).
A. Stained with CBB
B. Stained with glycoprotein

Figure 37. Electropherogram component patterns obtained from membrane aggregates formed at pH 9.0 in the presence of 0.001 M Mg^{++} (210 ug).
A. Stained with CBB
B. Stained with glycoprotein



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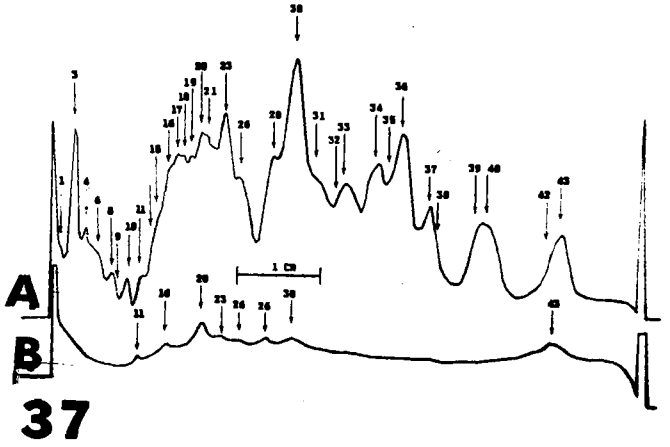


37

pare Figures 8A,B; 36A,B; and 37A,B).

Based on data given in Table IX, it can be seen that the percent composition of carbohydrate is greatest in aggregates formed in the presence of the higher magnesium concentration (0.01 M). Since we have demonstrated that proteins require more magnesium ions for aggregation than lipids, these data reinforce the conclusion that some carbohydrate exists in the membrane of this organism in a complexed state with proteins that is resistant to dissociation or solubilization by detergent; probably in a glycoprotein form.

Study of The Rate of Aggregation

To better understand the rate of reaggregation of disaggregated membrane components of M. lysodeikticus and, subsequently, to determine if the component species enter into aggregation at the same or different rates, aggregation experiments were performed (at pH 9.0 in the presence of 0.01 M magnesium) for various periods of time (ten, twenty, thirty-five, fifty and one hundred minutes and for twenty-four hours). After aggregation, each dialysis bag was placed in 2 liters of pre-chilled (4° C) distilled water and continually stirred for two hours at 4° C to stop aggregation. The resulting membrane aggregates were analyzed using SLS gel electrophoresis.

Aggregation was found to occur during all times tested. The pellets obtained upon sedimentation, however,

were not all the same size. Electrophoretic data shown in Figure 38 and summarized in Table X reveal that fewer component species were present in the ten minute aggregate (twenty-eight) than in aggregates formed after more extended periods of time (thirty-five to thirty-seven). Since species 23, 26 and 33 are the major ones present in the aggregate formed after only ten minutes of aggregation, it appears that these species enter into aggregation at a faster rate than all others. Further, the entrance of species 26 and 33 may be completed within the first half hour since these cease to be major components after twenty to thirty-five minutes aggregation. Unlike 26 and 33, the rate of entrance into aggregation of species 23 seems to be gradual and uniform since it is a major component in all the aggregates formed. Most of the major species (16, 20, 28, 30, 36 and 37) enter within thirty-five minutes (40 and 43 are exceptions). Therefore, aggregation occurs at a relatively rapid rate.

It was also noted that more sample (210 ug) was necessary for electrophoresis of these aggregates to obtain component stain intensities equal to those of aggregates formed during a period of twenty-four hours (150 ug electrophoresed) or of isolated whole membranes (120 ug electrophoresed). This information indicates that more phospholipid and carotenoid materials are present (on an equivalent weight basis) in aggregates formed during short periods of aggregation (ten and twenty minutes) than in ag-

Figure 38. SLS electrophoretic gel columns containing resolved membrane material components (CBB stained): membrane aggregates formed at pH 9.0 in the presence of 0.01 M Mg^{++}

A. isolated whole membrane (120 ug)
B. membrane aggregate formed during ten minutes aggregation (210 ug)
C. membrane aggregate formed during twenty minutes aggregation (210 ug)
D. membrane aggregate formed during thirty-five minutes aggregation (210 ug)
E. membrane aggregate formed during fifty minutes aggregation (210 ug)
F. membrane aggregate formed during one hundred minutes aggregation (210 ug)
G. membrane aggregate formed during twenty-four hours aggregation (150 ug)

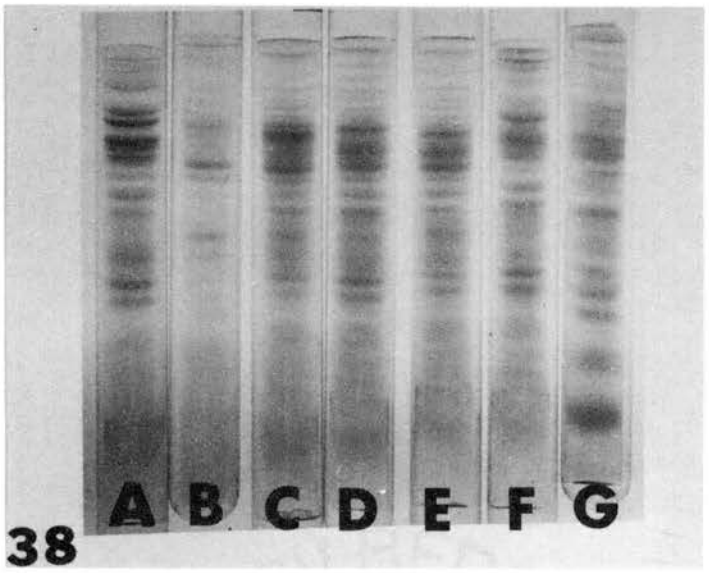

A
B
C
D
E
F
G
38

TABLE X

ANALYSIS OF THE COMPONENT SPECIES PRESENT IN MEMBRANE AGGREGATES OF *M. LYSODEIKTICUS* AGGREGATED FOR VARIOUS PERIODS OF TIME*

Situation	Aggregation Time (minutes)**					
	10	20	35	50	100	24 hrs
Approximate Number of Component Species Resolved (stained bands)***	28	35	37	37	36	37
Major Component Species Present in Membrane aggregates****		16	16	16	16	16
		17				
			20	20	20	20
	23	23	23	23	23	23
	26	26				
			28	28	28	28
			30	30	30	30
	33	33	33			
		36	36	36	36	36
		37	37	37	37	37
					40	40
						43

*Aggregation performed in 0.0025 M Tris buffer (pH 9.0) in the presence of 0.01 M magnesium.

**After aggregation, each dialysis bag was immediately placed in 2 liters of pre-chilled (4° C) distilled water and continually stirred using a magnetic stirrer for two hours at 4° C.

***Resolution accomplished by SLS gel electrophoresis.

****Based on intensity of staining (CBB).

gregates formed during longer periods or in whole membranes. Thus, not only does there exist a difference in rates which proteins enter into aggregation, but also a rate difference between lipid and protein appears to exist, with lipids entering at a faster rate. These data are in accordance with the study reported by Razin et al. (1967).

Membrane Protein Fractionation By Ammonium Sulfate Precipitation

To determine if any single protein or a group of proteins could aggregate into ordered sheets, solubilized membranes were fractionated by ammonium sulfate precipitation, and the various fractions placed under aggregating conditions. Membrane aggregates (stripped after aggregation prior to lyophilization) formed at pH 9.0 in the presence of 0.01 M magnesium were utilized as starting material since all proteins are present in these aggregates.

At first, 4 percent ammonium sulfate saturation was employed; however, no visual precipitation was observed after several hours at 4°C. Therefore, saturation was increased to 8 percent. Within two to three hours precipitation could be seen, and centrifugation yielded a silver, white pellet. After removal of this fraction (8 percent precipitate), saturation of the supernatant was increased to 25 percent, and after three hours the precipitate which formed was sedimented. Little, if any, precipitation was found to occur above 25 percent saturation.

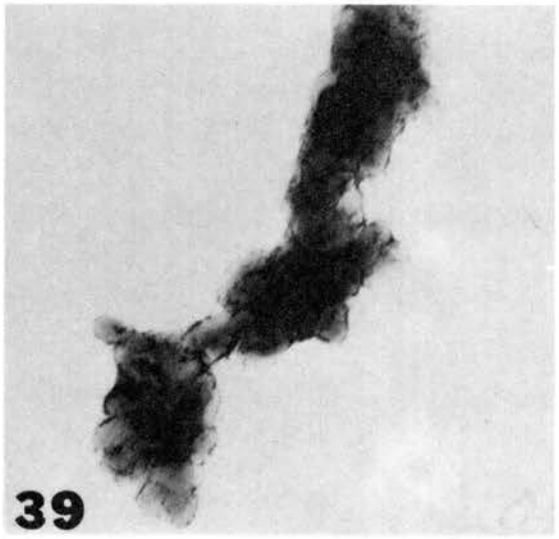
Following sedimentation, residual ammonium sulfate was removed by dialysis against distilled water and precipitated proteins were aggregated at pH 9.0 in the presence of 0.01 M magnesium. Pictorial data revealed that aggregation occurred using either fractions; however, ordered sheet structures were present only in the aggregate formed using the 8 percent fraction (Figures 39 and 40). Consequently, a difference in composition between these aggregates was thought to exist.

To determine if membrane proteins were actually fractionated using ammonium sulfate, electrophoretic SLS gel analysis was performed. The resulting component patterns of the precipitate aggregates revealed that indeed some fractionation occurred (Figure 41A,B,C); however, this involved only a small number of proteins (30 and 36 appear to be present almost solely in the 8 percent precipitate aggregate as well as being the major species present, whereas 28 and 37 are present almost solely in the 25 percent precipitate aggregate wherein they are the major species present). These data seem conclusive; however, stacking can be seen at the top of the gel column containing the electrophoresed 8 percent precipitate aggregate (Figure 41B). This indicates a problem with solubility, and thus, the pattern obtained may not be an actual representation of the proteins present in the precipitate. It should be pointed out, however, that this pattern is reproducible. Nonetheless, until the problem of stacking can be solved,

Figure 39. Membrane aggregates formed at pH 9.0 in the presence of 0.01 M Mg^{++} from the 8% ammonium sulfate precipitate. Negatively stained with uranyl acetate (47,300X).

Figure 40. Membrane aggregates formed at pH 9.0 in the presence of 0.01 M Mg^{++} from the 25% ammonium sulfate precipitate. Negatively stained with uranyl acetate (47,300X).

Figure 41. SLS electrophoretic gel columns containing resolved membrane components (CBB stained):
A. isolated whole membrane (120 ug)
B. material pictured in Figure 39 (180 ug)
C. material pictured in Figure 40 (180 ug)



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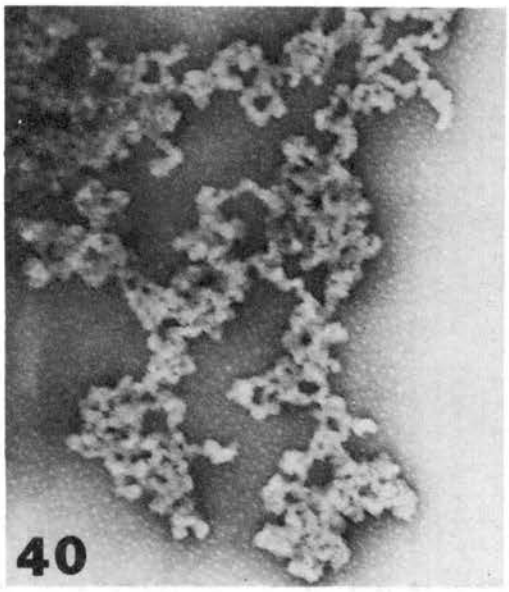

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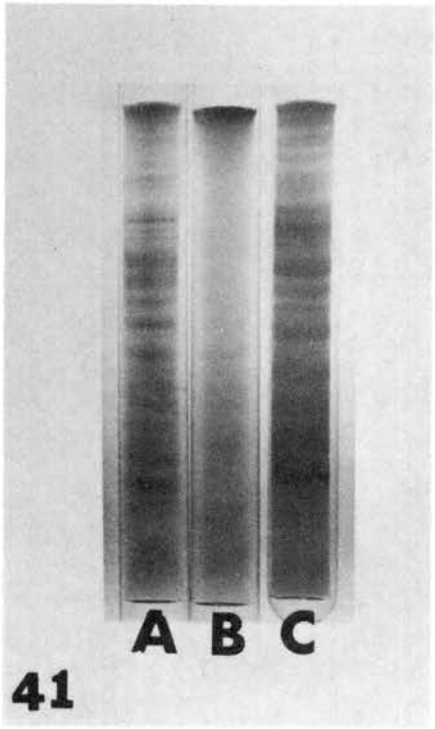

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definitive conclusions can not be made using these electrophoretic data.

Effect of Urea on Aggregation of Membrane Materials

Butler et al. (1967) were unable to disaggregate reaggregated membranes of M. lysodeikticus using 8 M urea; however, it was suggested that lack of solubilization by urea may have been due to the inability of urea to reach into the hydrophobic core of the basal membrane continuum rather than absolute lack of disaggregating ability. To aid us in determining if this is true, aggregation of several types of M. lysodeikticus membrane preparations was allowed to occur in the presence of 6 - 8 M urea.

Data shown in Figures 42, 43, 44 and 45 reveal that aggregation of the ordered sheet type occurs in urea using all the different types of membranes tested. The rate of aggregation, however, is much slower than usual (required three to seven days for completion as opposed to one day in the absence of urea).

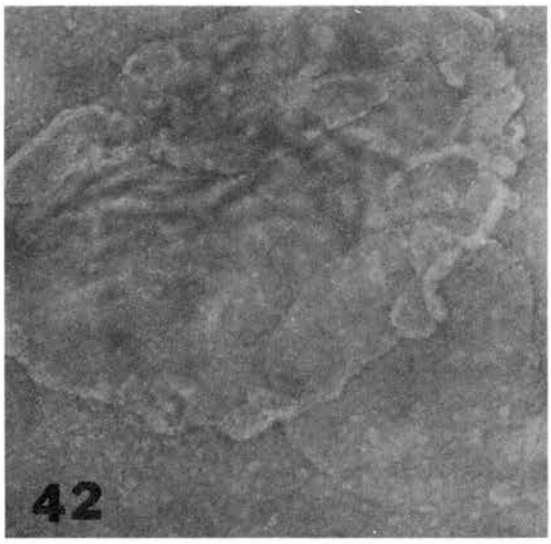
Overall, these data allow the conclusion that hydrogen bonds play a minor role in the structural organization of the membrane of this organism. It may be significant, however, that rate of aggregation is considerably slower in the presence of urea. This information indicates that although hydrogen bonds are not the primary mode of bonding involved in the structural organization of the membrane,

Figure 42. Membranous sheets formed at pH 9.0 in the presence of 0.01 M Mg^{++} and 6 M urea using disaggregated membrane components previously aggregated at pH 7.4 in the presence of 0.01 M Mg^{++}. Negatively stained with uranyl acetate (43,000X).

Figure 43. Membranous sheets formed at pH 9.0 in the presence of 0.01 M Mg^{++} and 6 M urea using disaggregated membrane components (stripped prior to disaggregation) previously aggregated at pH 9.0 in the presence of 0.01 M Mg^{++}. Negatively stained with uranyl acetate (86,000X).

Figure 44. Membranous sheets formed at pH 9.0 in the presence of 0.01 M Mg^{++} and 6 M urea using the 8% ammonium sulfate precipitate. Negatively stained with uranyl acetate (86,000X).

Figure 45. Membranous sheets formed as described in Figure 44 with the exception that isolated whole membranes were stripped prior to disaggregation. Negatively stained with uranyl acetate (86,000X).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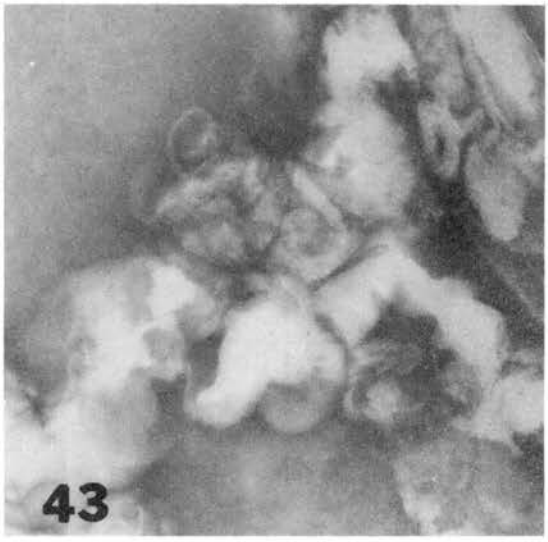
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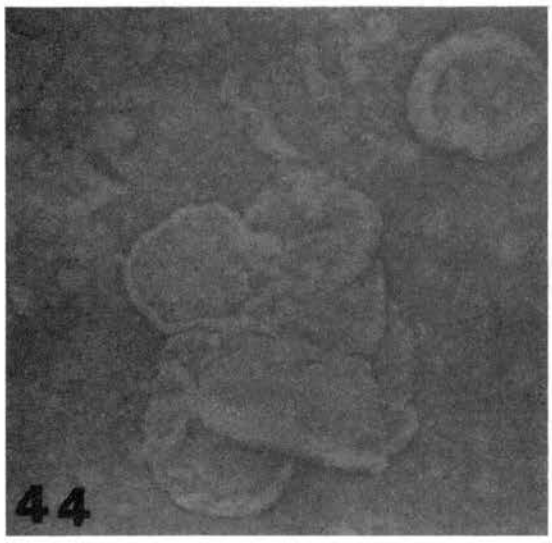
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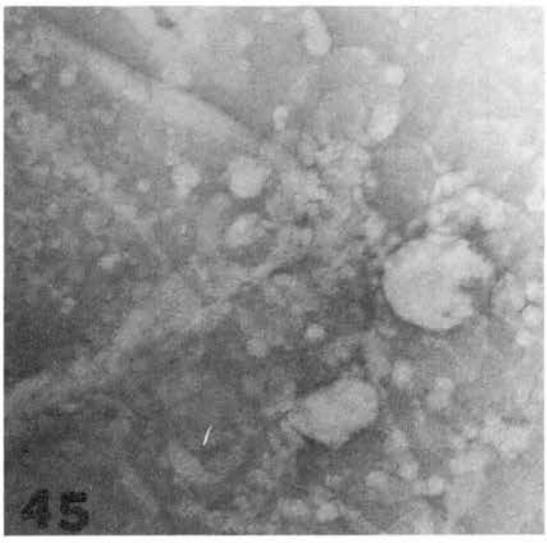
45

they may contribute to secondary and tertiary structure, and facilitate reaggregation of disaggregated membrane components.

Amino Acid Composition of Membrane Materials

Our data have shown that, regardless of the composition of the dialysis buffer during aggregation, only minor quantitative differences in protein composition can be demonstrated in fabricated membranous sheets. As shown in Table XI, composition of whole and aggregated membrane samples (pH 9.0 in the presence of 0.01 M magnesium) are closely similar having base-to-acid molar ratios of approximately 0.8. Percent composition of hydrophobic amino acids is also similar (approximately 63 percent). Further, alanine, glycine, leucine, glutamic acid, valine, aspartic acid and arginine are the major amino acids present. With the exception of the base-to-acid ratio, these data are in agreement with those previously reported for the membrane of M. lysodeikticus (Grula et al., 1967), and show, again, lack of cysteine in membrane proteins.

TABLE XI

AMINO ACID COMPOSITION OF *M. LYSODEIKTICUS* MEMBRANE MATERIALS*

Amino Acid	Molar Ratios	
	Whole Membrane	Aggregate Sample**
Alanine	8.00	6.41
Glycine	5.65	4.72
Leucine	4.81	4.69
Glutamic acid	4.15	3.67
Valine	3.58	3.44
Aspartic acid	3.30	3.36
Arginine	2.85	3.02
Phenylalanine	2.83	2.12
Threonine	2.65	2.48
Proline	2.58	2.50
Serine	2.20	1.92
Isoleucine	1.98	1.95
Lysine	1.83	1.87
Histidine	1.07	0.89
Tyrosine	1.00	1.00
Methionine	0.81	1.00
Ratio Basic/Acidic amino acids***	0.77	0.82
% Hydrophobic amino acids present****	64.0	62.0

*Samples were stripped and hydrolyzed in 6 N HCl at 105°C for eighteen hours prior to analysis using a Beckman Model 120 C amino acid analyzer.

**Membrane aggregate formed at pH 7.4 in the presence of 0.01 M magnesium for twenty-four hours at 25°C.

***Basic amino acids: histidine, lysine and arginine. Acidic amino acids: aspartic and glutamic acid. Acid ratio set at one.

****Hydrophobic amino acids: tryptophan, tyrosine, phenylalanine, cystine, methionine, proline, isoleucine, leucine, valine, alanine and glycine.

CHAPTER IV

DISCUSSION

This study was undertaken to determine if a single species of protein could be obtained from the membrane of M. lysodeikticus which would aggregate to form ordered membrane sheets. Ordered membrane structuring is defined as being in an organized or natural state; this was determined by appearance of the membranes using electron microscopy. If aggregates were other than ordered, they were considered to be denatured or unordered forms.

As demonstrated, no single species of protein could be obtained from any type of aggregated membrane of M. lysodeikticus in a pure form (determined by SLS gel electrophoresis). In addition, it could never be demonstrated that only one protein is present in great excess and to the exclusion of most, if not all, other proteins.

Based on polyacrylamide gel electrophoresis, the membrane is quite complex; much more so than previously envisioned when studied using ultracentrifugation (Butler et al., 1967). At least forty-three protein species are present and they possess MW values in the range from 10,000 to 100,000 daltons. To add to the complexity, selective staining utilizing a glycoprotein stain reveals that some pro-

tein molecules are closely associated with carbohydrate and exist probably as glycoproteins.

Even though no single protein was found to constitute the majority of proteins present in the membrane of M. lysodeikticus, it cannot be concluded that proteins are not responsible for ordered structuring. Our data could be interpreted to mean that ordered structuring is dependent on several proteins rather than any single species since about five proteins were found as major components in all membrane aggregates studied (Table IV).

The data and conclusions of others which relate to possible existence of structural protein in bacterial membranes present two opposing views. Mirsky (1969) fractionated membrane proteins of B. megaterium KM using ammonium sulfate and analyzed the fractions by the polyacrylamide gel system of Takayama et al. (1966). No single species of protein was found to comprise the majority of proteins present in the membrane of this organism. She concluded, therefore, that at the very least there must be a class of proteins which serve the function of providing a basic structure for the plasma membrane of bacteria. It is unfortunate that she did not utilize a detergent gel system for electrophoresis; however, we are in agreement with her general conclusions that many proteins are present in bacterial membranes.

Using Bacillus PP, a mutant of B. megaterium KM, Patterson and Lennarz (1970) fractionated the membrane pro-

teins utilizing the electrophoretic gel systems of Weber and osborn (1969) and Takayama et al. (1966). They concluded that a single species of protein, having a MW of 32,000, comprises at least ninety percent of the total protein present in the cell membrane of this mutant. The same protein exists as a relatively minor component in the parent organism. It was not concluded that this protein is structural protein; however, the implied meaning seems clear.

One hypothesis for the general structuring of membranes that has found general acceptance is that put forward by Danielli and Davson (1935) and slightly modified by Robertson (1959). These investigators concluded that the basic structuring of membranes is a bimolecular leaflet (bilayer) of phospholipids, the nonpolar regions of which are inwardly oriented perpendicular to the plane of the membrane. The external surface of the bilayer is covered by and ionically bonded to a layer of protein. If this is the general type structuring for membranes, there is little need to search for "structural protein(s)" since lipids rather than protein(s) make up the basal membrane continuum.

Grula et al. (1967) presented data obtained utilizing the membrane of M. lysodeikticus that is not in accord with the phospholipid bilayer theory. If phospholipid is present between two layers of protein and serves as the basal continuum of the membrane, it was reasoned that the familar trilaminar structuring should not be present in membrane

sheets formed from stripped proteins. Because trilaminar membrane sheets were formed using stripped "subunits", they suggested that phospholipids may be present as a protective superstructure on the top and bottom of structural membrane protein. It was also suggested that phospholipids could serve to "anchor" other proteins which have enzymatic activity within or on the membrane.

Some experiments which relate to the presence or absence of a phospholipid bilayer in the membrane of M. lysodeikticus were performed in this study. Of prime importance was the experiment wherein phospholipids and carotenoids were removed by stripping with the acetone reagent and then placed under optimal conditions of aggregation for membrane sheet formation. Precipitation of these components occurred; however, no ordered sheet structures were observed (Figure 35). Such information suggests that at least some type of protein is essential to allow fabrication of an ordered membrane sheet. On the other hand, the need also for phospholipids as structural components is indicated by experiments wherein it was shown that a large decrease in aggregation (seventy-three percent) occurs when phospholipids and carotenoids are stripped away from membrane proteins (Table VII). Unfortunately, denaturation of membrane proteins by the acetone stripping reagent cannot be ruled out in any of these type experiments.

Since phospholipids and carotenoids cannot by themselves aggregate into ordered membrane sheets and because

proteins stripped of most phospholipids aggregate very poorly, my data may indicate that membrane structuring is determined by both protein and phospholipid molecules. Experiments designed to determine the effects of magnesium ion concentration on aggregation revealed that entry of phospholipids into aggregation is less dependent on magnesium than protein (Table VI). Such information suggests that phospholipids may combine with a limited number of proteins and provide a basic structure for entry of other proteins into aggregation.

In further support of this idea, it was observed that all protein species do not enter into aggregation at the same rate; instead, some enter completely within the first few minutes, some gradually throughout and others only after prolonged periods of aggregation. Of particular interest was the finding that the five protein species which are major components in isolated whole membranes, as well as in all ordered membrane aggregates studied, enter into aggregation within the first few minutes. If these five proteins are involved with phospholipids in providing the basic structure for the membrane, it would certainly be expected that they would enter into aggregation quickly. Therefore, it is entirely conceivable that the final ordered aggregate is gradually constructed, with fabrication dependent initially on some type of combination between certain proteins and phospholipids rather than on either alone.

In summary, approximately forty-three species of protein, some as glycoproteins, are present in the membrane of M. lysodeikticus. None, however, constitute the majority of proteins present either in isolated whole membranes or membrane aggregates fabricated under the conditions utilized in this study. A small number of proteins (five) are always present in ordered membrane structures; however, since they enter into aggregation quickly (within the first few minutes), they may aid in providing a basic structure for membrane fabrication. Since the amount of aggregation is greatly increased when phospholipids are present, phospholipids may serve to stabilize membrane proteins. However, since phospholipids enter into aggregation at a faster rate than protein, it is also possible that their function is to provide a basic structure for entry of some proteins into a membrane sheet; possibly in the form of a bilayer. Thus, ordered structuring could not be demonstrated to be completely dependent on either protein or phospholipid; however, protein appears to be more important since ordered membrane sheets can be fabricated with most phospholipids and carotenoids removed. Overall, this study has demonstrated that the membrane of M. lysodeikticus is a very complex entity, whose basic structure may be provided not by a single structural protein or phospholipid bilayer but by a small number of proteins plus phospholipids; possibly intermingled with one another.

Several points can be mentioned which concern the pos-

sibility that protein-detergent micelles are present in our aggregation system. Using radioactive detergent (SDS), Rottem et al. (1968) reported that dialysis for three to four days at 4° C against 1:20 B-buffer removes over ninety-nine percent of the detergent. Therefore, it seems reasonable that our procedure of precipitation and dialysis would also remove at least ninety-nine percent of the detergent; however, precise proof is lacking on this point.

In addition, data have been reported (Shapiro and Maizel, 1969) which indicate that the bond between detergent and protein molecules is not very strong. During detergent gel electrophoresis, it was found that none of the proteins of adenovirus retained detectable nonexchangeable ^{35}S-labeled SDS. Overall, these data argue strongly against the existence of significant amounts of micelles consisting of proteins and detergent in our aggregation system.

Although disaggregation of the membrane of *M. lysodeikticus* was accomplished utilizing detergent, we observed (Table X) that all protein species do not enter into aggregation at the same rate. Since proteins bind detergent (SDS) stoichmetrically (Reynolds and Tanford, 1970), it seems reasonable that all species of protein would enter into aggregation at approximately the same rate if only protein-detergent micelles are present and if aggregation is dependent on this form of unit or material. As a result, electropherogram component patterns of isolated whole membranes and membrane aggregates would be almost identi-

cal, i.e., component species would have approximately the same relative stain intensity in membrane aggregates as observed in isolated whole membranes. As shown in this thesis, component patterns of membrane aggregates and isolated whole membranes are not identical. Therefore, it seems reasonable to conclude that protein-detergent micelles do not exist, at least in great numbers, in our aggregation system.

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