MEMBRANE REAGGREGATION STUDIES USING THE GRAM-NEGATIVE BACTERIUM *ERWINIA* SPECIES

By

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ERWINIA SPECIES

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CHAPTER I

INTRODUCTION

Grula and Grula (1964) have suggested that the cell membrane initiates cell division in Erwinia species. In order to investigate possible membrane involvement, it was necessary to develop a method to isolate the membrane from this Gram-negative organism.

Membranes from animal cells, subcellular organelles, and bacteria that have no cell wall, such as Mycoplasma, can be isolated with relative ease. Weibull (1953) observed that the enzyme lysozyme would hydrolyze and remove the cell wall from Bacillus megaterium, thus forming cells (protoplasts) for which the membrane was the sole peripheral limiting structure. Protoplasts formed in this manner will lyse under hypotonic conditions yielding cell membranes that can be separated readily from cytoplasmic debris by centrifugation. This type of procedure can be successfully utilized with Gram-positive bacteria which have cell walls with a relatively uniform macromolecular composition (mucopeptide).

The Gram-negative bacteria have a much more complex cell wall composition. Kellenberger and Ryter (1958) in studying ultrathin sections of Escherichia coli made the definite statement that the outer triple layered structure was a portion of the wall, not the membrane. Murray (1963) made a much more detailed study of the cell wall of the Gram-negative organism Spirillum serpens. He confirmed the work of Houwink (1953) who described a hexagonal packed wall layer located

outside all other layers of the wall normally seen in thin sections of whole cells. Murray and Houwink considered this layer to be lipoprotein of the cell wall. Murray reported that treatment with 1% sodium lauryl sulfate (SLS) solubilized this material. Murray, Steed, and Elson (1965) showed that the innermost layer of the cell wall of E. coli is the mucopeptide. McIntire, et al. (1967) have shown that sodium dodecyl sulfate (SDS) disaggregates lipopolysaccharide (LPS) of E. coli. The electron microscope studies of Murray, Steed, and Elson (1965) and the chemical data of Weidel, Martin, and Frank (1960) indicate that the Gram-negative cell wall has an outer layer of lipoprotein covering the lipopolysaccharide, a protein layer, and an innermost layer of mucopeptide.

Although these models are essentially correct, the exact structural arrangement of the lipoprotein and the lipopolysaccharide is still in question because the surface antigens of some bacteria as well as phage receptor sites are the lipopolysaccharide of the cell wall.

This short review of the structural make-up of the Gram-negative cell wall is included to point out the difficulty in the selective removal of the various cell wall layers that would be necessary to isolate cellular membranes in a manner analogous to that used with Micrococcus lysodeikticus (Butler, Smith, and Grula 1967).

In addition to having a complex make-up, the Gram-negative wall is lipid-containing; this tends to make it somewhat chemically similar to the cell membrane. In an endeavor to circumvent the necessity for the selective removal of the numerous and chemically diverse layers of the wall, we attempted isolation of the structured and associated portions

of the membrane by use of the self-recognition or reaggregation phenomenon.

The idea that biological membranes are made up of elementary particles or subunits was originated by Green and Hechter (1965). They stated that such lipoprotein units were responsible for the structural and functional role of animal mitochondria. These elementary particles have been dissociated and reconstituted by numerous workers (Green and Hechter, 1965; King, 1962; Hatefe, Haavik, Fowler, and Griffiths, 1962). Green, et al., (1961) state that reconstitution occurs primarily through hydrophobic bonding.

Razin, Morowitz, and Terry (1965) showed that treatment with detergent (SLS) solubilized the peripheral membrane from Mycoplasma laidlawii into a subunit form. When these subunits were dialyzed in the presence of sodium chloride, magnesium ions, and a reducing compound (2-mercaptoethanol) typical membrane structures were readily formed and could be observed using the electron microscope. The detergent solubilization was thought to dissociate the membrane into lipoprotein subunits because only one symmetrical schlieren peak was obtained using the analytical ultra-centrifuge. Subsequent experimentation by Engelman, Terry, and Morowitz (1967) and Rottem, Stein, and Razin (1968) (the former using density gradient centrifugation and the latter using polyacrylamide disc gel electrophoresis) has shown that detergent treatment separates lipid from the protein. Therefore the detergent solubilization does not cause the formation of lipoprotein subunits; rather they appear to be protein subunits.

Comprehensive studies concerning the reaggregation phenomenon have been conducted by two groups of workers. Reaggregation of Mycoplasma

membrane subunits has been studied by Rottem, Stein, and Razin (1968) and the reaggregation of M. lysodeikticus membrane subunits by a group in our laboratory (Butler, 1967; Butler, Smith, and Grula, 1967; Grula, Butler, King, and Smith, 1967).

Reaggregation studies using Mycoplasma revealed that for reaggregation to occur the detergent used to solubilize the membranes must be removed and, also, a divalent cation must be present. It was found too that once reaggregation of this material had occured it could not be solubilized by dialysis against ethylenediaminetetraacetic acid (EDTA). Reaggregates formed by monovalent cations (Na^{+}) were solubilized when they were resuspended in deionized water. The authors, Rottem, Stein, and Razin (1968), stated that although both monovalent and divalent cations could neutralize negative charges allowing the subunits to reform, only the divalent cations could lend the needed additional stability to the reaggregates by the formation of salt bridges between negatively charged groups on adjacent molecules. Studies concerning the Mg++ concentration revealed that the lipid to protein ratio varied with variation in the Mg++; low concentration of Mg++ yielded high lipid to protein ratios. It was also found that reaggregates formed at pH 3.5 required significantly less Mg++ than those formed at pH 7.4.

Studies of reaggregation in M. lysodeikticus showed the conditions for reaggregation were very similar to those reported for Mycoplasma. It was further shown that neither NaCl nor the reducing agent present in the dialysis medium of Razin, Morowitz, and Terry (1965) were required. The amount of reaggregation was found to be directly proportional to temperature up to 37°C. Although whole and reaggregated membranes were similar chemically and morphologically, they did show differences in

their ability to be solubilized by various detergents. Treatment with urea, guanidine, and incubation at various pH values from 3.0 to 10.0 did not cause disaggregation.

A very significant finding of these studies was that membrane from which the lipids had been extracted could be disaggregated and subsequently reaggregated in the absence of lipids. This indicates that the membrane structure is due to protein associations and that lipids are not necessary for proper orientation of membrane subunits. To further support this point, reaggregates formed from delipidized membranes (stripped) showed a typical trilaminar structure when viewed in cross section.

As there has been little success in attempting to isolate membranes from Gram-negative bacteria without considerable cell wall contamination, we attempted to approach this problem from a different direction. Using a system involving detergent solubilization of the cell envelope, we were assured of little or no mucopeptide contamination, because it is not detergent soluble (Grula, Smith, and Grula, 1965). The fact that bacterial membranes have been shown to reaggregate into structurally similar forms in Mycoplasma laidlawii and Micrococcus lysodeikticus, led us to believe that such a system could also be developed to produce membrane sheets from the Gram-negative organism Erwinia species.

CHAPTER II

MATERIALS AND METHODS

Test Organism

The organism used in these studies was a species of Erwinia. The organism, although not easily identifiable, is a soft rot-producing organism closely resembling Erwinia caratovora (Grula, 1960a). Stock cultures were maintained alternately (24 hour intervals) on nutrient agar and nutrient agar plus 1% glucose at room temperature and stored at 4°C. All inoculations were made using a 24 hour nutrient agar slant culture.

Medium and Growth

Cells were grown in a defined medium, (Grula, 1960b), consisting of the following compounds per 100 ml: glucose (300 mg) added aseptically, $MgSO_4 \cdot 7H_2O$ (3 mg), K_2HPO_4 (174 mg), KH_2PO_4 (136 mg), aspartic acid (420 mg) compounded separately and adjusted to pH 6.8, and trace minerals. The trace mineral solution was made separately and added to give the following amounts per 100 ml of medium: $CaCO_3$, 10.0 ug; $CuSO_4 \cdot 5H_2O$, 1.0 ug; $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$, 50.0 ug; KI, 1.0 ug; $MnSO_4 \cdot H_2O$, 2.0 ug; MoO_3, 1.0 ug and $ZnSO_4 \cdot 7H_2O$, 5.0 ug, (pH 6.8).

This medium, without the glucose, was sterilized in an autoclave for 15 minutes at 15 pounds pressure. Glucose was autoclaved separately

(stock solution, 2.5 g per 100 ml) for 10 minutes at 12 pounds pressure and added to the medium aseptically.

The medium was inoculated with cells grown on nutrient agar slants at room temperature for 24 hours. The cells were washed twice with 0.85% sterile saline and adjusted to a final optical density of approximately 0.1. One ml of this cell suspension was inoculated into 100 ml of medium made up in 250 ml Erlenmyer flasks. Cultures were incubated on a rotary shaker at 25°C for approximately 16 hours. At this time the cells were in late log phase and had an optical density of about 0.35.

Isolation of Envelopes and Preparation of Detergent Soluble Subunits

After 16 hours of growth, cells were harvested by centrifugation, washed once with distilled water, and resuspended to a very high density in Q-buffer composed of Tris 0.0025 M and 0.008 M NaCl adjusted to pH values varying from 4.1 to 10.3 (same pH as used for subsequent dialysis). At this point the cells were broken either by sonic disruption (multiple five second bursts at maximum output using a Branson sonifier; each burst being followed by one minute cooling in an ice bath), or by mechanical breakage utilizing an X-PRESS. Mechanical breakage was accomplished by placing a heavy suspension of cells in the pre-chilled X-PRESS and allowing the suspension to freeze, then forcing it through the unit by using a Carver laboratory hydraulic press. Breakage occurs at about 20,000 lb/sq. inch. The resulting particulate fraction (envelope) was collected by centrifugation at 54,500 x g for 30 minutes (Spinco Model L Centrifuge). The envelope fraction was washed twice with Q-buffer. Subunit formation was accomplished by adding

2 ml of 0.3 M SLS to envelopes from 100 ml of culture medium suspended in 18 ml of Q-buffer.

After 15 minutes at room temperature, the preparation was placed at 4°C overnight. At this temperature much of the SLS precipitates. The precipitated SLS, mucopeptide, and any other substances which were not soluble in SLS, were removed by centrifugation at 54,500 x g for 30 minutes. A flow diagram for this procedure is presented in Figure 1.

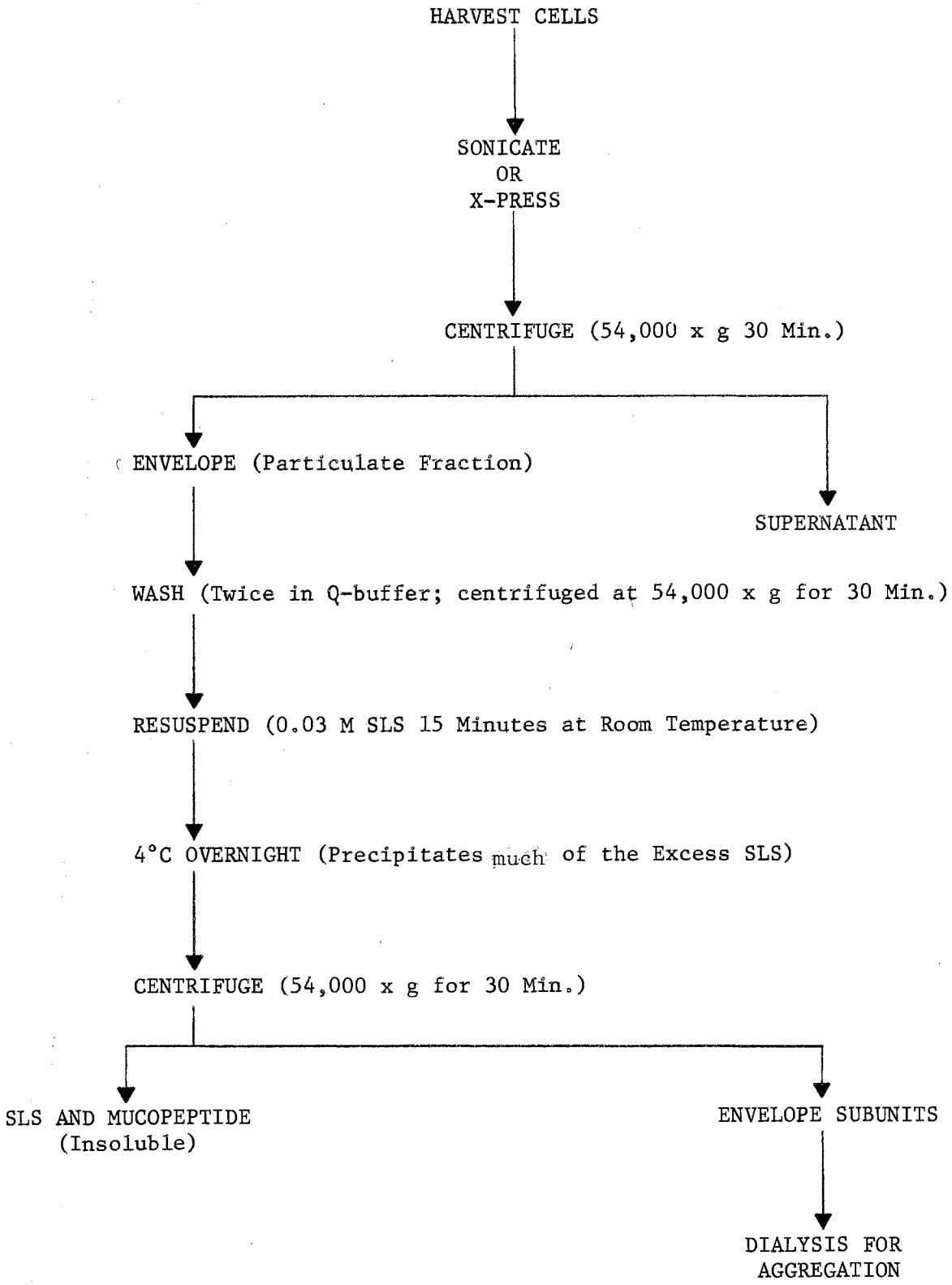
Reaggregation Technique

The subunits formed by SLS treatment were placed in dialysis tubing and dialyzed using a buffer volume to sample volume ratio of 100:1. The dialysis buffer contained Tris, 0.0025 M; NaCl, 0.008 M; $MgCl_2$, 0.01 M. Dialysis was continued at 4°C for four to seven days until turbidity was observed in the bag. At this time the reaggregates were collected by centrifugation at 54,500 x g for 30 minutes.

Phospholipid Isolation

Either whole cells or reaggregates were suspended in 20 ml of methanol. The air space in the container was thoroughly flushed with nitrogen gas to prevent air oxidation and tightly stoppered with a screw cap. This suspension was heated at 55°C for 30 minutes. After heating, the suspension was allowed to cool and two volumes of chloroform were added. The containers were again flushed with nitrogen and allowed to stand overnight at 25°C. The chloroform-methanol solution was filtered through a millipore filter to remove particulate matter. The filtrate was then washed twice with two volumes of 2 M KCl and once with one volume distilled water and the aqueous layer removed each time by

Figure 1. Flow diagram for the preparation of detergent soluble envelope subunits. All centrifugation done in a Spinco Model L centrifuge.


HARVEST CELLS
SONICATE
OR
X-PRESS
CENTRIFUGE (54,000 x g 30 Min.)
ENVELOPE (Particulate Fraction)
SUPERNATANT
WASH (Twice in Q-buffer; centrifuged at 54,000 x g for 30 Min.)
RESUSPEND (0.03 M SLS 15 Minutes at Room Temperature)
4°C OVERNIGHT (Precipitates much of the Excess SLS)
CENTRIFUGE (54,000 x g for 30 Min.)
SLS AND MUCOPEPTIDE
(Insoluble)
ENVELOPE SUBUNITS
DIALYSIS FOR
AGGREGATION

aspiration. The washing procedure was accomplished by gently inverting the containers 20 times, thereby minimizing emulsion formation. The 2 M KCl washes removed protein present in the filtrate and also removed the methanol. The resulting chloroform solution containing the phospholipids was concentrated by evaporation under vacuum and the concentrated solution was stored under nitrogen.

Phospholipid Chromatography

Phospholipids were identified using thin-layer chromatography as described by Stahl (1960). Silica Gel G (Merck) was spread on glass plates (20 x 20 cm) using a Brinkmann spreader to a thickness of 250 microns and dried at 25°C. The plates were heat-activated at 100°C for one hour and stored under vacuum. The thin layer chromatograms were developed in chloroform-methanol-water (65:25:4, v/v/v). The spray reagents used were: 1) Rhodamine G (0.001%) for general lipid detection followed by observation under ultra-violet light (Luzzati and Husson, 1962); 2) Ninhydrin (ninhydrin, 0.5% in 95% acetone) for detection of amino-containing lipids (these chromatograms were heated at 100°C until the appearance of blue color); 3) The method of Hanes and Isherwood (1949) was used for detection of phosphate-containing lipids; and 4) The Dragendorf reagent spray of Wagner, Hornahammer, and Wolfe (1961) was used for detection of choline-containing lipids.

Disc Gel Electrophoresis

The method used was a modification of a procedure described by Neville (1967). This system was used without benefit of a stacking gel. The gel has the following composition: 7% acrylamide, 0.2%

N, N'-methylenebisacrylamide, 0.58 M acetic acid, 0.0025 M KOH, and 9 M urea.

Gels were polymerized with the aid of the following catalysts: 0.0004% riboflavin, 0.05% ammonium persulfate, and 0.08% N, N, N', N' tetramethylethylenediamine. Gels were exposed to a fluorescent light for one hour to aid in polymerization.

Both upper and lower reservoirs of the electrophoresis apparatus were filled with 10% acetic acid (v/v). The lower electrode served as the cathode (Rottem and Razin, 1967). Electrophoresis was carried out at a constant current of 2.5 ma per tube for about one hour. Methyl green served as the marker dye.

Pre-running of gels to remove excess catalyst was done by electrophoresing the gels at 5 ma per tube for one hour with the lower electrode being the cathode. The upper and lower 10% acetic acid solutions were both discarded after this pre-run step.

Prior to electrophoresis, the proteins were solubilized using phenol-acetic acid-water: 2.0:1.0:0.5, w/v/v (Rottem and Razin, 1967).

Gels were stained with 1% naphthol blue black for one hour and destained by operating the electrophoresis unit with both compartments filled with 10% acetic acid at 5 ma per tube (polarity reversed) until all excess stain was removed.

Electron Microscopy

Thin Slice Preparation

Pelleted reaggregates were fixed with 6.25% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for two hours at 4°C, washed in 0.1 M

sodium cacodylate buffer, cut into blocks, and post-fixed for two hours with one percent osmium tetroxide in Michaelis acetate veronal buffer (sodium acetate, 0.238 M; sodium veronal, 0.143 M; sodium chloride, 0.58 M; pH 6.1) (Razin, Morowitz, and Terry, 1965). The blocks were allowed to set overnight in 0.5% uranyl acetate in acetate veronal buffer. The blocks were then washed in acetate veronal buffer and dehydrated using the folowing ethanol series: 25%, 50%, 75%, 90%, 100%, and 100% for 15 minutes at each step. The ethanol was removed by twice immersing the blocks in 100% propylene oxide for 15 minutes.

The blocks were then allowed to set in 50% Araldite (Robson, 1964) and 50% propylene oxide for two hours, 75% Araldite and 25% propylene oxide overnight, and in 100% Araldite for 24 hours. All the above manipulations were carried out in tightly stoppered vials. Final embedding was accomplished by placing the blocks in medicine capsules (No. 00) and filling the capsules with 100% Araldite. The capsules were allowed to stand at room temperature for 24 hours, then placed in an oven at 62°-65°C for 72 hours. Sections were cut using a Sorvall MT-2 ultramicrotome. Sections were picked up on 400 mesh copper grids, stained for 15 minutes with 0.4% (aqueous) lead citrate and methanol (1:1, v/v), then dried with filter paper.

Negative Stain

Small drops of reaggregate suspensions were placed on 200 mesh copper grids which were coated with collodion and covered with a thin carbon film. The suspension was left on the grids for 30 to 90 seconds, blotted with filter paper, and a drop of 2% uranyl acetate was placed on

the grid. After 30 seconds the stain was drawn off using filter paper, and the grid allowed to dry.

All specimens were examined using an RCA EMU-3G electron microscope operated at 100 Kv.

Prior to deposition on grids, samples must be washed twice with distilled water to remove salts which interfere with staining and viewing.

Gel Filtration Column Chromatography

Both Sephadex G-75 and G-200 were used. In both cases columns were prepared in accordance with the recommended procedure of the manufacturer (Sephadex--Gel Filtration Theory and Practice).

The buffer used with the G-200 Sephadex was 0.0025 M Tris-HCl (pH 7.4) containing 0.001 M SLS. The fluid used with G-75 Sephadex was distilled water containing 0.001 M EDTA.

Lipopolysaccharide (LPS) Isolation

The isolation procedure used was an adaptation of the phenol method of Westphal and Luderitz (1954). The envelopes obtained from 100 ml of culture medium were suspended in 10 ml of a mixture of phenol and water (45% w/v). This biphasic system was placed in a water bath maintained at 70°C for 30 minutes with periodic mixing. The sample was then removed and allowed to separate into two phases. This was followed by centrifugation. The aqueous phase was removed and ethanol was added to a final concentration of 70%. After standing overnight at 4°C the LPS formed a white opaque precipitate which was collected by centrifugation.

Chromatography

The LPS was hydrolyzed for liberation of sugar components using 4 N HCl for four hours at 100°C in a sealed evacuated container.

Descending paper chromatography was done using strips of Whatman Number 1 paper approximately 24 inches long. The solvent used was ethlyacetate : pyridine : water (8:2:1). The strips were cut in such a manner as to form a point at one end, thus allowing the solvent to run off the end of the strip in an unhindered manner. Chromatography was carried out for a period of 18 hours. The Redfield two-dimensional system was also used for sugar identification. The first directional solvent was methanol : water : pyridine (80:20:4). The second directional solvent was t-butanol : methylethylketone : water : diethylamine (40:40:20:4).

The sugar-spray reagent was composed of 2-aminobiphenyl, 1.69 grams; oxalic acid, 0.9 grams; glycerol, 5 ml; water, 10 ml; and acetone, 84 ml. Color development was achieved by heating the chromatograms at 100°C for 5 to 10 minutes.

Molecular Weight Determination

The molecular weight determination was done by a method of Thorun and Mehl (1968), using both 8% and 12% acrylamide gel concentrations.

CHAPTER III

RESULTS AND DISCUSSION

Envelope Isolation

Before reaggregation studies could be done, it was necessary to obtain isolated envelopes essentially free of cytoplasmic contamination. The first method of cell breakage tried was sonic disruption. Although this is an adequate method, Butler (1967) reported that isolated cell membranes could be disaggregated into a subunit form by sonic disruption. It was therefore decided to try a less severe method; mechanical breakage using an X-PRESS. A comparison of the protein patterns of envelopes prepared by these two methods is shown in Figure 2.

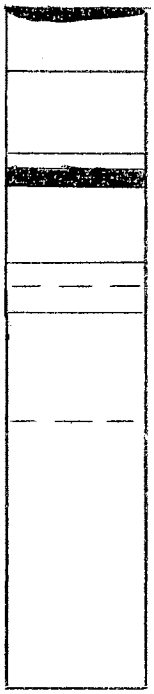
It can be seen that the envelopes obtained by mechanical breakage show more protein bands. Corresponding bands could also be observed in some of the envelope preparations obtained by sonic disruption. However from these and other experiments, it was decided that mechanical breakage was probably a more gentle process and that the variable protein patterns obtained from sonic disruption were caused by solubilization of envelope-associated proteins. In addition to yielding more reproducible protein patterns, mechanical breakage gives a greater percentage of cell breakage. It was later found that envelopes formed by use of the X-PRESS gave greater amounts of reaggregation when compared to envelopes

Figure 2. Comparison of protein profiles of envelopes prepared by sonic disruption and the X-PRESS. Diagrams of gels two times actual size. Dashed lines indicate very faint bands.

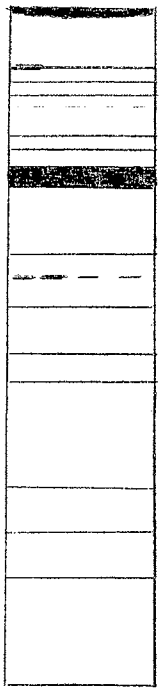
A. Envelopes prepared by sonic disruption

B. Envelopes prepared by using the X-PRESS

A



B



prepared by sonic disruption. Therefore, most of the envelopes for the following experiments were isolated using the X-PRESS.

Reaggregation of Subunits Obtained from Isolated Envelopes

Reaggregation of membrane subunits was reported by Razin, et. al. (1965) for Mycoplasma laidlawii. Subsequently, Butler, et al. (1967) reported a somewhat modified system for the Gram-positive bacterium M. lysodeikticus. The initial system used in these studies was based on the one developed by Butler, et al. (1967). The dialysis medium consisted of Tris, 0.0025 M; NaCl, 0.008 M; and $MgCl_2$, 0.01 M; with the pH adjusted to a value of 7.4 with HCl.

Using this medium for reaggregation, subunits obtained by SLS treatment of isolated envelopes were dialyzed for 4 days with a buffer change after the first 24 hours. After 24 hours, a small amount of turbidity could be observed in the dialysis bag. At the end of four days, a large amount of insoluble material had formed.

To determine whether the insoluble material in the bag consisted of reaggregated membrane sheets, the material was collected by centrifugation, washed twice with distilled water, and observed using the electron microscope (Figure 3). Negative-staining of this preparation indicated that sheets were formed under our conditions of dialysis and that they were similar in appearance to either whole or reaggregated membrane sheets obtained from M. lysodeikticus.

Chemical Treatments

Reaggregates obtained from M. lysodeikticus sediment as a transparent pellet; however, they are yellow in color due to the presence of carotenoid pigments. The reaggregates obtained from the non-pigmented Erwinia envelopes are not as transparent. A series of treatments were tried with a twofold purpose: 1) to obtain a more uniform (clear) appearing pellet, and 2) to gain an insight into the possible types of chemical bonding which hold the reaggregated sheets together. It was already known that hydrophobic bonding was of major importance because SLS causes dissociation of the membrane. Of the three treatments--high ionic strength (NaCl, 1 M for 20 minutes at 25°C), a hydrogen bond breaking reagent (urea, 8 M for 20 minutes at 25°C), and a chelating agent (EDTA, 0.1 M for 20 minutes at 25°C)--only the first yielded a clear pellet. It was later found that reaggregation at alkaline pH values also yielded a relatively clear pellet. Electron micrographs (Figure 4) showed that only urea had any effect on the appearance of the reaggregated sheets. Although the sheets appeared disrupted, they were not solubilized. Therefore hydrogen bonding, although possibly involved, is not the major chemical type of bonding in the structural association of membrane subunits in Erwinia species. Reaggregates were also treated with trypsin (1% in 0.06 M phosphate buffer, pH 8.0 for 60 minutes at 37°C) and pepsin (0.5% in 0.05 M glycine HCl buffer, pH 2.2, for 60 minutes at 37°C). Trypsin treatment caused no observable effect; however pepsin treatment yielded sheets that appeared significantly more transparent. To test whether this was an effect of pepsin, or the low pH of the incubation mixture, reaggregates were incubated as before, but with the pepsin omitted. Subsequent electron microscopy revealed that

the observed effect was the result of low pH (Figure 5). This low pH effect could be due to an alteration of sheet structure or possibly to the removal of associated material from the sheets. Reaggregates were also delipidized (stripped) using acetone-ammonia (Fleischer, Fleischer, and Stoeckenius, 1966) to see if phospholipids had any structural role. Figure 6 shows that removal of phospholipids does not cause dissolution of the reaggregated sheets.

To further test the importance of phospholipids in the reaggregation process, isolated envelopes were stripped, disaggregated with SLS and then dialyzed (pH 9.1) for 5 days. Electron microscopy revealed that sheet formation occurred although the majority of the sheets were considerably smaller than normal.

Hexagonal Packed Material (HPM)

During the electron microscopic examination of reaggregates treated with 1 M NaCl, small amounts of fine structured material were observed. Upon subsequent photographic enlargement, this material appeared to be made-up of subunits arranged in a symmetrical hexagonal pattern (Figures 7, 8, and 9). Similar hexagonal packed subunits have been observed in Spirillum sp., Rhodospirillum rubrum, Halobacterium halobium, Lampropedia hyalina, and Escherichia coli (Howink, 1953, Salton and Williams, 1954; Houwink, 1956; Chapman, et al. 1963; Murray, 1963; Fishman and Weinbaun, 1967). All of these investigators observed this structure in the cell wall of Gram-negative bacteria. Fishman and Weinbaun, Murray and Houwink suggested that this structure represented the lipoprotein of the cell wall.

Munoz, Freer, Ellar, and Salton (1968), in studying isolated membranes from M. lysodeikticus, observed a similar structure which they

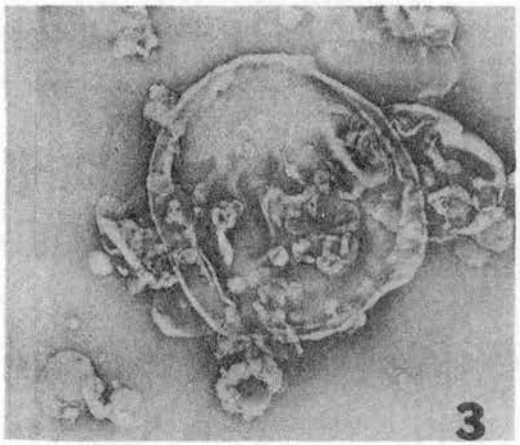
Figure 3. Reaggregated membrane sheets (51,000X)

Figure 4. Effect of urea on reaggregated membrane sheets (51,000X)

Figure 5. Effect of low pH on reaggregated membrane sheets (51,000X)

Figure 6. Effect of acetone extraction on reaggregated membrane sheets (51,000X)

All negatively stained with 2% uranyl acetate.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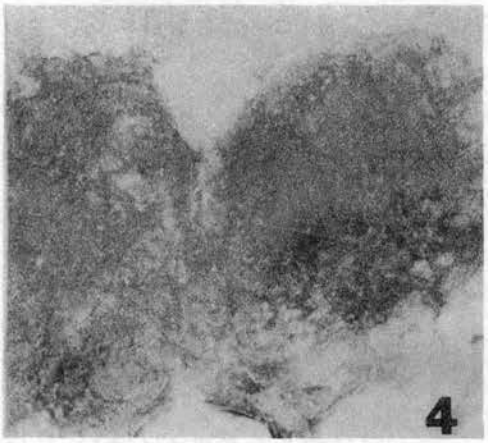
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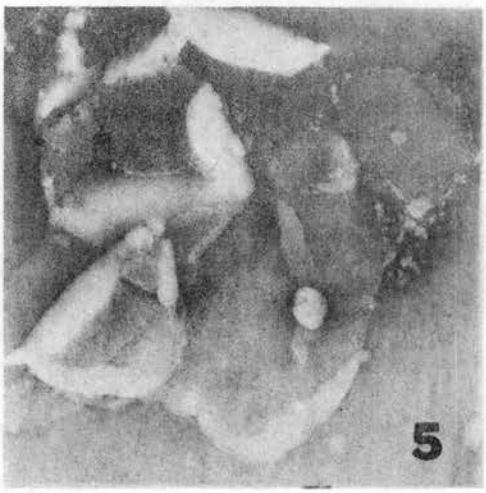
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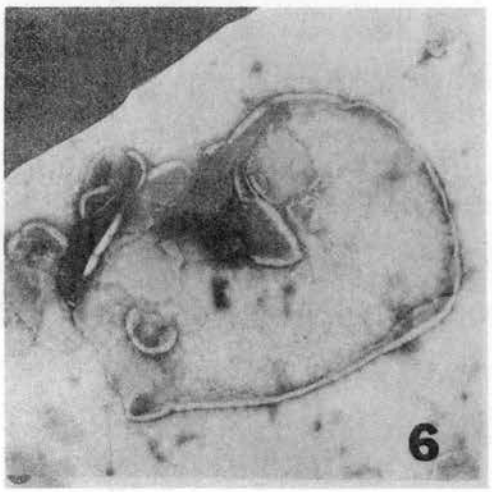
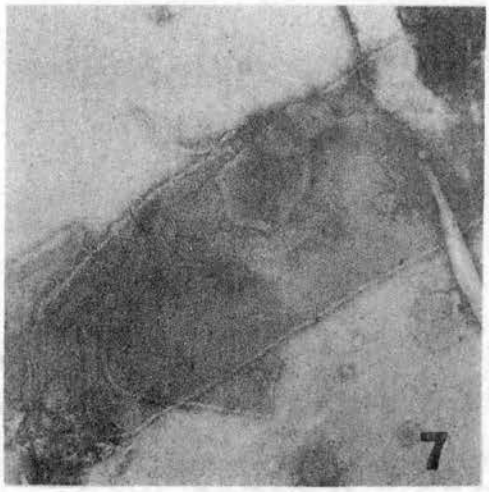

6

Figure 7. Hexagonal packed material (51,000X)

Figure 8. Hexagonal packed material (146,000X)

Figure 9. Diagrammatic representation of hexagonal packed material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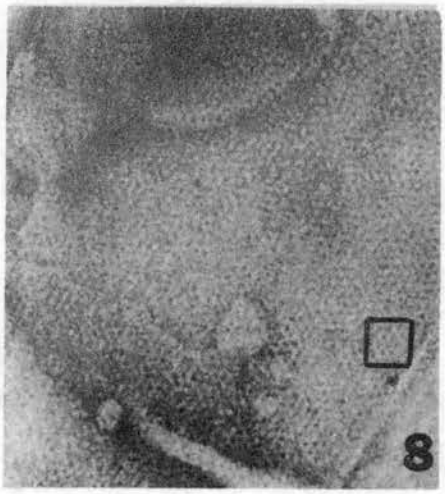
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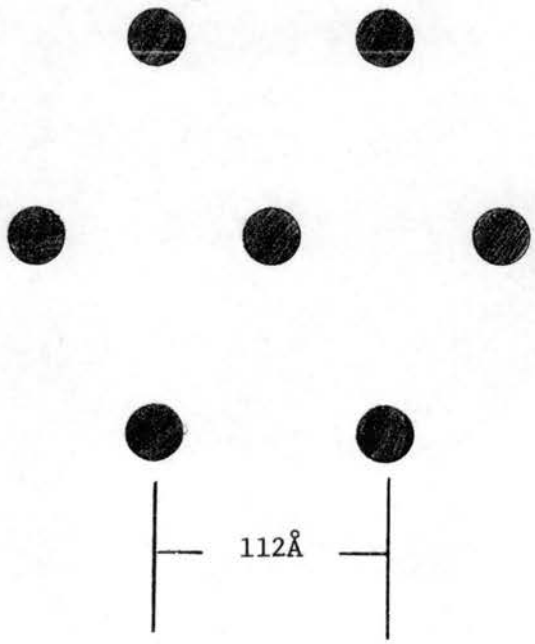

112Å

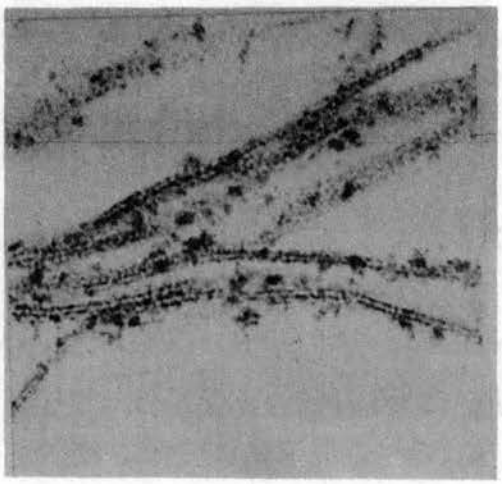
showed to be subunits of the membrane-associated enzyme adenosinetriphosphatase (ATPase). Although the photograph shown (Figure 7) is of reaggregates treated with 1 M NaCl, small amounts of HPM have been observed under all conditions with the exception of reaggregates exposed to an environment of low pH values. Preliminary tests have indicated that no ATPase activity is associated with the reaggregated membrane sheets.

At this time, enough data are not available to conclude whether the HPM that is observed in reaggregates (either carried over intact or possibly reformed from subunits by a reaggregation phenomenon similar to that found with membranes) composes a portion of the cell wall of Erwinia, is ATPase, or is some entirely different cellular component. With the limited information available it would seem most likely that the HPM is the lipoprotein of the cell wall. Observations of reaggregates indicate that the HPM constitutes only a small portion (less than 1%) of the total reaggregate. Further critical study of HPM would necessitate development of a procedure for selective isolation of this material.

Morphology of Thin Sections

Thin-sections of reaggregates treated with 1 M NaCl (Figure 10) reveal the typical "trilaminar" (dark-light-dark) staining that is characteristic of biological membranes. This pattern is also observed in untreated reaggregates. The cross-sectional width is 72-75Å which agrees closely with reports of other authors for membranes (Salton, 1967).

Figure 10. Thin slice of reaggregated membrane sheets (197,800X)



Conditions for Reaggregation

The initial reaggregation system of Razin, et al. (1965) was simplified by Butler, et al. (1967). This simplified version was the starting point for this study.

The dialyzing fluid was composed of Tris, 0.025 M; NaCl, 0.008 M; and $MgCl_2$, 0.01 M, the pH adjusted to 7.4 with HCl. Various $MgCl_2$ concentrations were tested to determine which level gave the maximum amount of reaggregation (Table I).

TABLE I

EFFECT OF Mg++ CONCENTRATION ON REAGGREGATION

$MgCl_2$ Concentration	O.D. at 540 mu
0.00	.00
0.001 M	.01
0.01 M	.31
0.03 M	.71

Dialyzed five days at 4°C

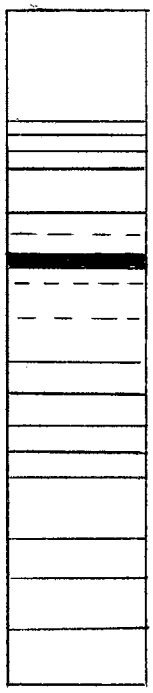
Results given in Table I indicate the need for a divalent cation; an increase in $MgCl_2$ concentration causes a larger amount of reaggregation. To determine if there was any difference in the nature of the material reaggregated at different $MgCl_2$ concentrations, samples were analyzed using disc gel electrophoresis (Figure 11).

Figure 11. Effect of $MgCl_2$ concentration on the protein profiles of reaggregated membrane. Dialysis medium at pH 9.1 with no buffer change. Dashed lines indicate very faint bands.

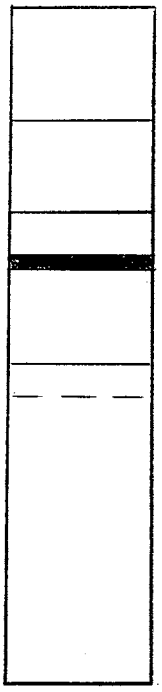
A. Reaggregates formed in dialysis solution containing $MgCl_2$ (0.03 M).

B. Reaggregates formed in dialysis solution containing $MgCl_2$ (0.01 M).

A



B



It was observed that the higher concentration of $MgCl_2$ yielded a greater number of protein bands. This could mean that there is a difference in ability to bind Mg++ and therefore certain proteins reaggregate or precipitate, depending upon the amount of Mg++ available.

To determine if ionic strength of the buffer had any effect on reaggregation, various levels of NaCl were tested. (Table II).

TABLE II

EFFECT OF NaCl CONCENTRATION ON REAGGREGATION

NaCl Concentration	O.D. at 540 mu
0.00	0.48
0.008 M (control)	0.60
0.5 M	0.03

Dialyzed five days at 4°C and washed twice with deionized water before reading.

The 0.5 M NaCl level caused a large amount of precipitation which was water soluble and removable during washing. The results as given in Table II show that a high concentration of NaCl inhibits reaggregation. This could be caused by the great excess of Na^+ ions blocking sites required for Mg++ ions, or the protein might be precipitated before forming stable reaggregates. It was also observed that NaCl, although not necessary for reaggregation, is slightly stimulating at the 0.008 M concentration.

Precise temperature studies were not done, but it was noted that reaggregation was much more rapid at 25°C than at 4°C.

Table III shows the effect of pH on the reaggregation process.

TABLE III

EFFECT OF pH OF THE DIALYSIS MEDIUM ON REAGGREGATION

pH of Buffer	O.D. at 540 mu
4.1	0.95
5.3	0.64
7.4	0.60
8.5	0.35
9.6	0.37

Dialyzed five days at 4°C and washed twice with deionized water before reading.

Examination of negatively-stained preparations using the electron microscope revealed that reaggregated sheets were obtained at all pH values tested. Values of pH as high as 10.3 or as low as 2.3 allowed formation of similar appearing sheets. Disc gel electrophoretic analysis of similar material was also accomplished and these results are shown in Figure 12.

These data demonstrate that a greater number of proteins are present when reaggregation occurs at low pH than at alkaline pH values. The pattern obtained at a low pH (4.1) is essentially the same as that obtained when subunits are permitted to aggregate at high Mg^{++} (0.03 M) concentrations.

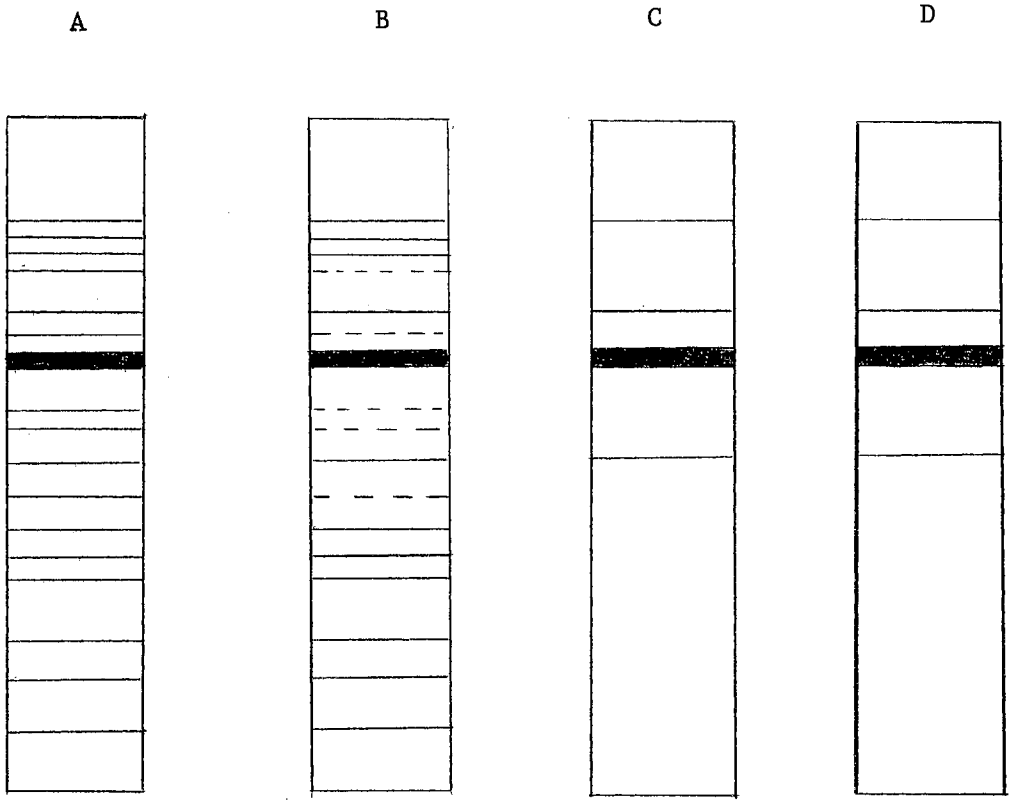
Figure 12. Protein profiles of reaggregated membrane prepared at varying pH values. Dashed lines represent very faint bands.

A. Reaggregated at pH 4.5

B. Reaggregated at pH 7.5

C. Reaggregated at pH 9.3

D. Reaggregated at pH 10.3


A
B
C
D

The increased number of proteins present when reaggregation is accomplished in a low pH environment could be caused by isoelectric point precipitation of envelope-associated proteins. The idea of negative-charge neutralization causing the proteins to become insoluble is supported somewhat by the fact that increased Mg++ concentrations would also be expected to neutralize negative charges. Figure 13 further demonstrates the similarity between the protein pattern obtained from isolated envelopes and that obtained from reaggregates.

Chemical Composition of Reaggregates

Reaggregated sheets were treated with lysozyme to see if these structures were composed of cell wall mucopeptide. Lysozyme had no observable effect on reaggregate sheets as judged by observation using the electron microscope. For a more rigorous check on the presence of mucopeptide, reaggregates were hydrolyzed (4 N HCl for 4 hours) and amino sugars were assayed for by the method of Rondell and Morgan (1955). A small amount of amino sugar was found; however, muramic acid could not be detected. Failure to detect muramic acid indicates that little or no mucopeptide is present in the reaggregated sheets.

The amino sugar was tentatively identified as glucosamine. This is in agreement with the finding of Grula and Grula (1964) who analyzed isolated envelopes of Erwinia species.

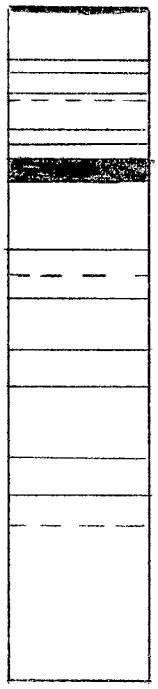
We also wished to determine if a significant amount of lipopoly-saccharide (LPS) was present in the reaggregated material. The procedure of Westphal and Luderitz (1954) was used. It was observed that the major reducing sugar in the LPS of Erwinia is glucose. The same isolation procedure applied to reaggregates also gave a detectable

Figure 13. Comparison of protein profiles from isolated envelopes and reaggregated membranes. Dashed lines represent very faint bands.

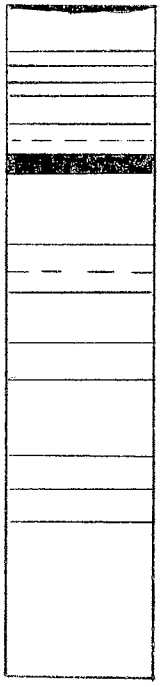
A. Protein profile of isolated envelopes

B. Protein profile of reaggregated membrane (pH 9.1, 0.03 M Mg^{++})

A



B



amount of glucose. Although some LPS is present in the reaggregates, it comprises less than 1% of the total dry weight of the reaggregated material. Glucosamine could not be detected in the isolated LPS. Studies of supernatant fluid after centrifugation of reaggregated sheets revealed that a considerable amount of carbohydrate was present (Molisch test). This leads one to believe that the LPS is disaggregated by detergent; however, very little reaggregates under our conditions.

The results of these experiments indicate that the reaggregate material contains very little or no mucopeptide and only a slight amount of LPS. The only other indication that the reaggregates contain anything other than membrane is the presence of HPM, and very little of this is present. Thus, we have concluded that the major components making up a reaggregated sheet is material derived from the plasma membrane.

The following chemical analyses were undertaken to further substantiate this conclusion and to compare these findings with the findings of other workers.

Protein, using the procedure of Lowry, et al. (1951), was found to make-up 40% of the dry weight of the reaggregated membrane. Solubilized reaggregated membrane show an abnormally low absorption at 280 mu, (primarily for tyrosine and tryptophan residues) and protein assay of stripped lyophilized reaggregated membrane that should be at least 90% protein, reveals that the assay is only about 66% efficient. Similar observations have been made using reaggregated membrane from M. lysodeikticus. Amino acid analyses of reaggregated membrane from M. lysodeikticus reveal that reaggregated membrane is quite low in aromatic amino acids, particularly tyrosine. Considering the previously mentioned limitations, the protein content of reaggregated membrane is reported as 40-60%.

Lipids were extracted using the method of Folch, Lees and Stanley (1957) and found to comprise 32% of the reaggregated membrane by weight. The phospholipids isolated from reaggregated membrane correspond to those found in whole cell extractions as judged by thin-layer chromatography (Figure 14). Also detected in lipid extracts of whole cells and reaggregated membrane was a compound that migrated to an RF of .92-.94 in iso-octane : ethyl ether (100:30) (spray detection using Rhodoamine B, 0.5%, and observed under ultraviolet light).

Figure 15 shows the UV absorption spectrum of this compound(s). Absorption maxima are at 217 mu, 221 mu, 277 mu, 285 mu, and 325 mu. Salton and Smith (1967), using this technique, have isolated similar compound(s) from isolated membrane of M. lysodeikticus and concluded that they are menaquinones.

To determine the amount of nucleic acid present, reaggregated membrane was extracted using trichloroacetic acid (TCA, 5% in a boiling water bath for 20 minutes). The results of this experiment are given in Table IV. It can be seen that when compared to isolated envelopes, the reaggregated membrane contains little or no 260 mu absorbing material.

TABLE IV

COMPARISON OF 260 mu ABSORBING MATERIAL IN ENVELOPES AND REAGGREGATED MEMBRANE

Sample	O.D. 260 mu	O.D. 280 mu	ug/ml NA*
Envelope TCA Extract	1.05	0.765	60.5
Reaggregate TCA Extract	0.00	0.135	00.0

*NA: Nucleic acid ug amounts taken from nomograph (Cal. Biochem.).

Figure 14. Phospholipid patterns of whole cell extracts and of reaggregated membrane extracts. Reaggregates formed at pH 7.4, 0.01 M Mg^{++}. Spots represent phosphate-containing lipids.

*PTG = phosphatidyl glycerol

**PTE = phosphatidyl ethanolamine

***DPTG = diphosphatidyl glycerol

Solvent system: Chloroform-methanol-water (65:25:4)

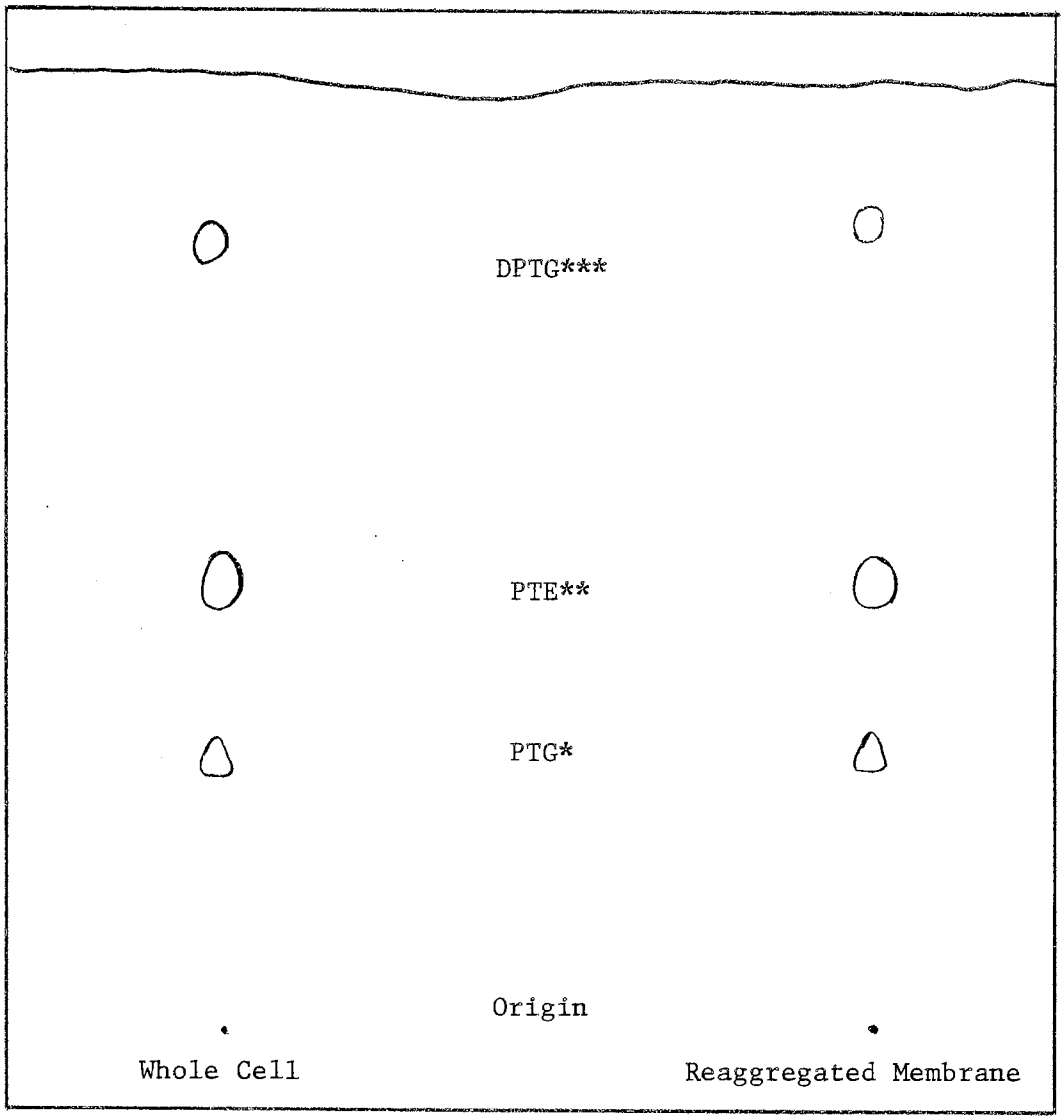
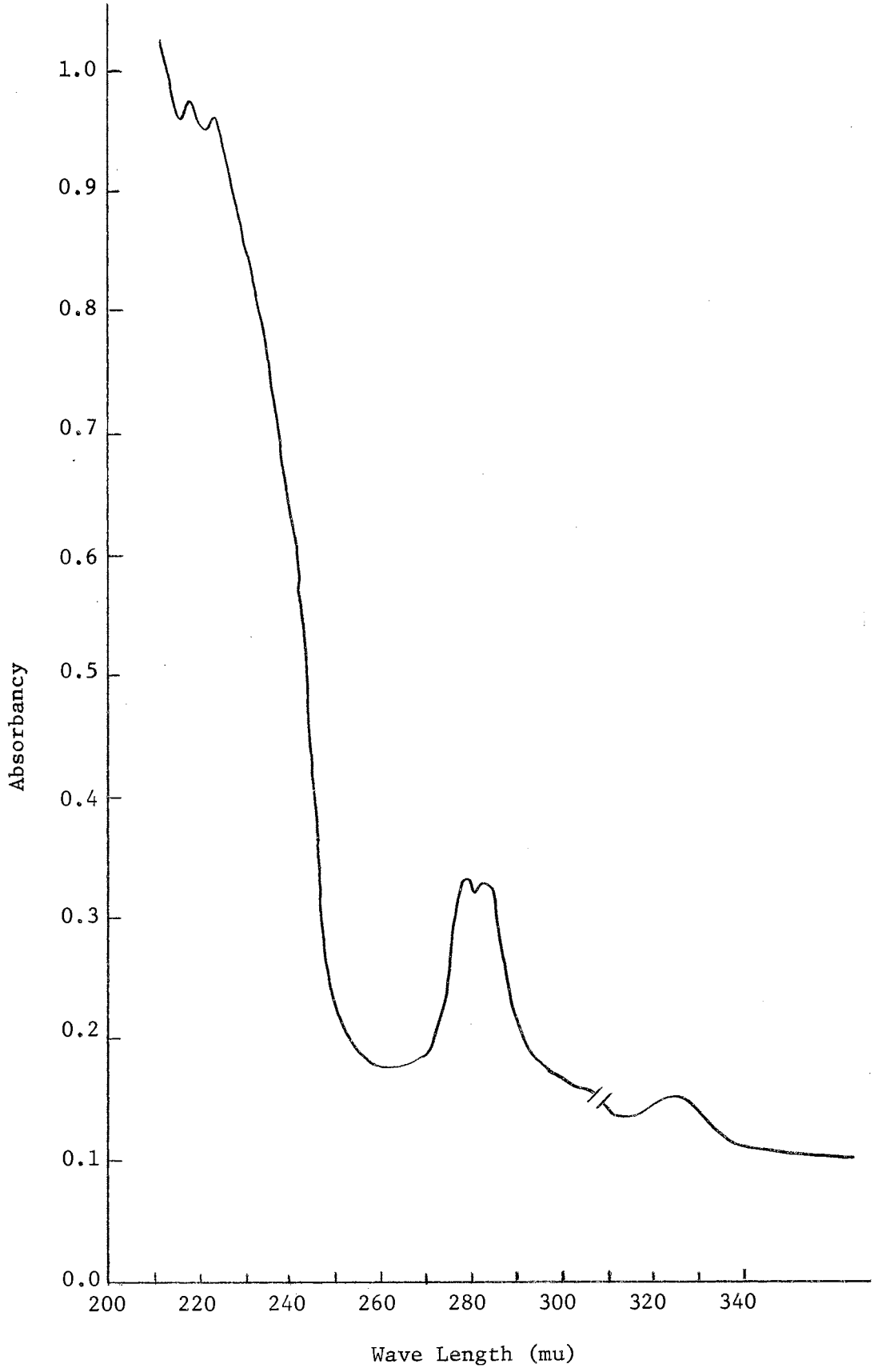

DPTG***
PTE**
PTG*
Origin
Whole Cell
Reaggregated Membrane

Figure 15. Absorption spectra of lipid extractable compound. Sample dissolved in iso-octane. Break mark (/ /) represents necessity to dilute sample in order to resolve absorption maxima at lower wave lengths. Readings were made using a Cary recording spectrophotometer.


Absorbancy
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
200
220
240
260
280
300
320
340
Wave Length (mu)

Isolation of Structural Protein

It was found that lyophilization does not affect protein profiles on polyacrylamide gels of reaggregated membrane sheets or isolated envelopes and that they are readily solubilized in phenol : acetic acid : water (2:1:0.5). The protein profiles obtained after various treatments of the reaggregates and from the various conditions of dialysis indicated that reaggregation at pH 9.1 with limited Mg++ (no buffer change during dialysis), resulted in significant purification of the one predominant species of protein. A comparison of Figure 16, with Figure 12 (gel B), shows that reaggregation at pH 9.1 with limited Mg++ results in formation of membrane sheets containing only one major and three minor bands of protein as compared to control reaggregates (pH 7.4) wherein one major, ten minor and five very faint bands of protein are present. It was hypothesized that if the membrane has a single protein species that serves as the basis for the membrane structure (structural protein), the one major protein consistently observed would be the most logical choice.

We attempted to obtain an estimate of the molecular weight of the major protein present in the reaggregated membrane (pH 9.1 dialysis with limited Mg++). The disc gel method of Thorun and Mehl (1968) was used. This method involves comparison of migration rates in gels of different acrylamide concentrations (8% versus 12% acrylamide). The results of this experiment are shown in Figure 17. The molecular weight for this protein is estimated to be 35,000 $\pm$ 5,000.

Figure 16. Effect of high pH and limited Mg^{++} on the protein profile of reaggregated membrane. Reaggregated at pH 9.1 without buffer change.

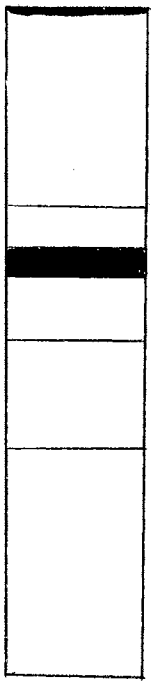
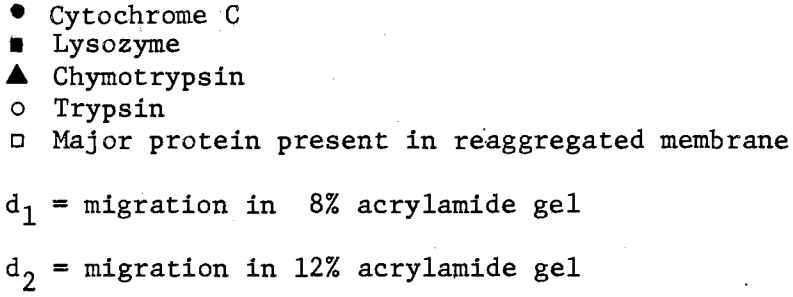
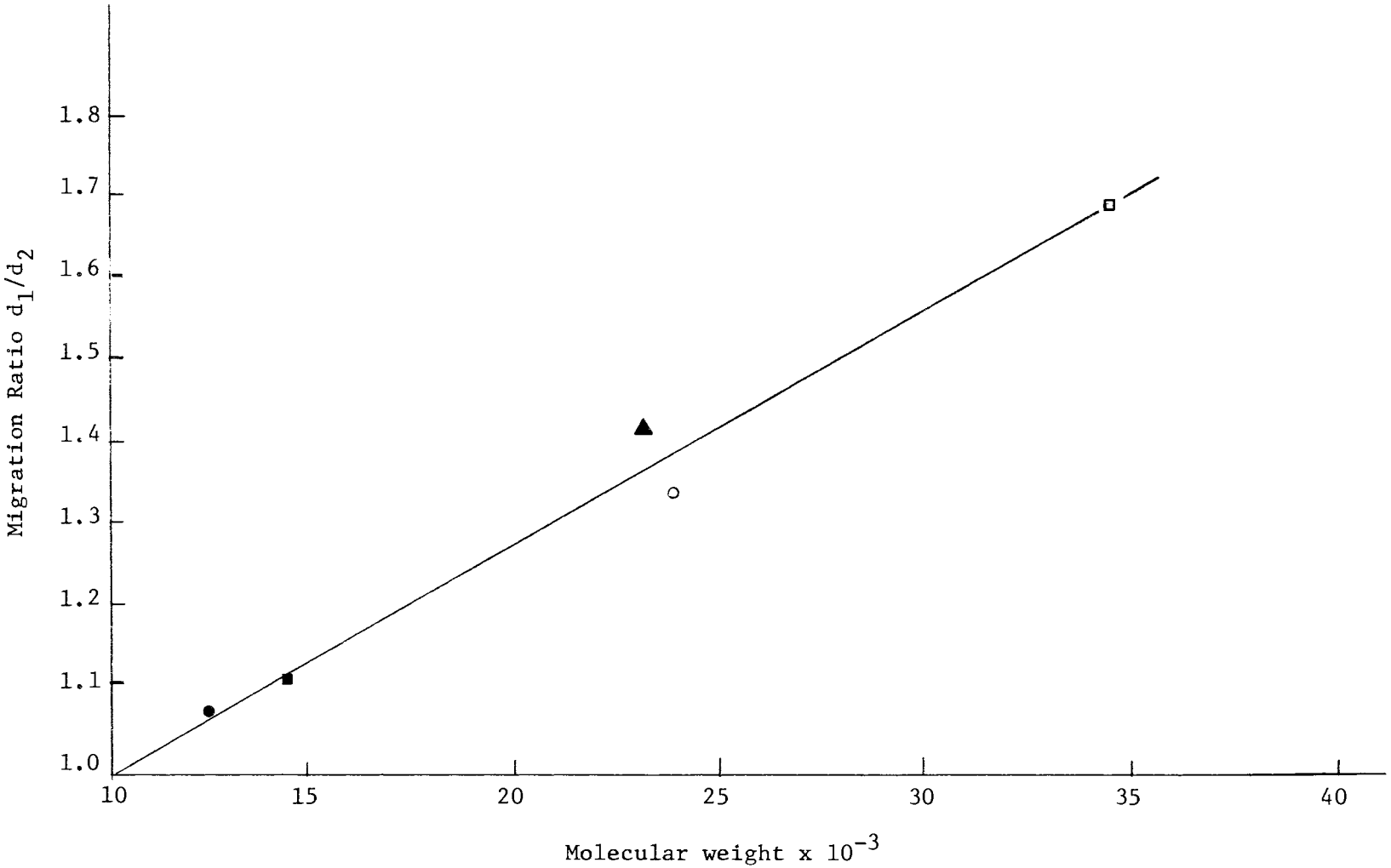


Figure 17. Determination of the molecular weight of the major protein species present in reaggregated membranes.




Migration Ratio d_1/d_2
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
10
15
20
25
30
35
40
Molecular weight x 10^{-3}

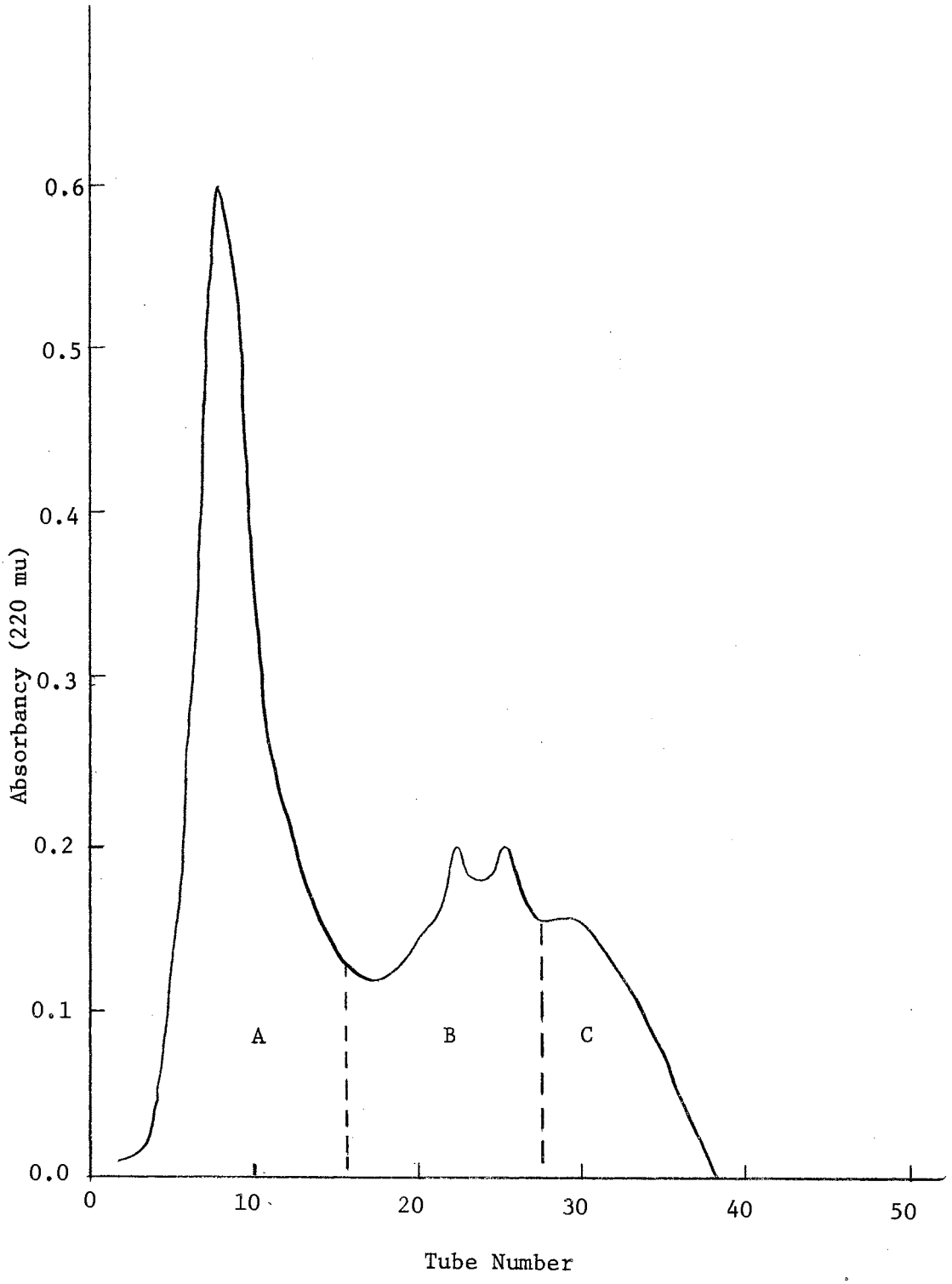
Gel Filtration Chromatography

In an initial attempt to isolate structural protein from envelopes, a sample was solubilized in SLS and applied to the top of a Sephadex G-200 column. Samples (100 drops) were collected using an automatic fraction collector and the absorption of each sample was read at 220 and 280 mu (220 mu was a more sensitive assay and was used routinely). The results of this experiment are shown in Figure 18. Fractions A, B, and C were placed under dialysis conditions (pH 7.4). The resulting reaggregates were harvested and observed using the electron microscope. Normal appearing sheets were obtained from all these fractions. Also disc gel electrophoresis was done for each fraction. Our results indicated that this is not a satisfactory method for selective isolation of proteins from reaggregates simply because little or no fractionation resulted.

It was felt that the detergent drastically affected the properties of the Sephadex; therefore, we decided to utilize a method that did not involve the use of SLS.

Envelopes were disaggregated using SLS and dialyzed in the usual manner, but with Mg^{++} omitted from the dialyzing medium. In this way it was hoped that the detergent would be removed, but reaggregation would not occur. It was found that even with numerous buffer changes, sufficient detergent remained to affect gel filtration chromatography. Therefore, the remaining SLS in the sample was removed by passing the material through a G-25 Sephadex column. The sample was then concentrated by dialysis against 10% carbowax 6000 and applied to the top of a column containing G-75 Sephadex equilibrated with 0.001 M EDTA.

Figure 18. Sephadex G-200 gel filtration chromatography of envelope subunits. Each tube contains 100 drops (about 2 ml). First peak corresponds to the void volume. A, B, and C represent samples pooled for reaggregation.


0.6
0.5
0.4
0.3
0.2
0.1
0.0
Absorbancy (220 mu)
A
B
C
0
10
20
30
40
50
Tube Number

(EDTA was used to prevent reaggregation of the subunits during the time they remained on the column.) Figure 19 shows the elution profile obtained from this experiment. The samples from Peak B were pooled and placed under reaggregation conditions (pH 9.1). Figure 20 shows the results of disc gel electrophoretic analysis of the resultant reaggregated material.

Preparative Disc Gel Electrophoresis

In an attempt to approach isolation of structural protein using another procedure, preparative disc gel electrophoresis was also utilized.

Reaggregated membrane sheets obtained by dialysis at pH 9.1 with limited Mg++ were solubilized and applied to one dozen pre-run gels and electrophoresed for 45 minutes. The migration rates of the various protein bands were previously determined and the section of the gels corresponding to the major protein band were removed by cutting them out of the gels. Individual sections were then placed in de-staining tubes which had dialysis tubing attached to their ends. The apparatus was run for two hours (5 ma per tube) to move the protein out of the gels. Samples collected in this manner were pooled and then dialyzed against distilled water, after which they were lyophilized. The results of this isolation procedure are shown in Figure 21. Although large amounts of structural protein cannot be isolated utilizing this procedure, the material isolated did show only one band upon subsequent electrophoresis.

Figure 19. Sephadex G-75 gel filtration chromatography of envelope subunits. Each tube contains 100 drops (about 3.5 ml). The first peak (A) corresponds to the void volume. All samples in Peak B were pooled for reaggregation.

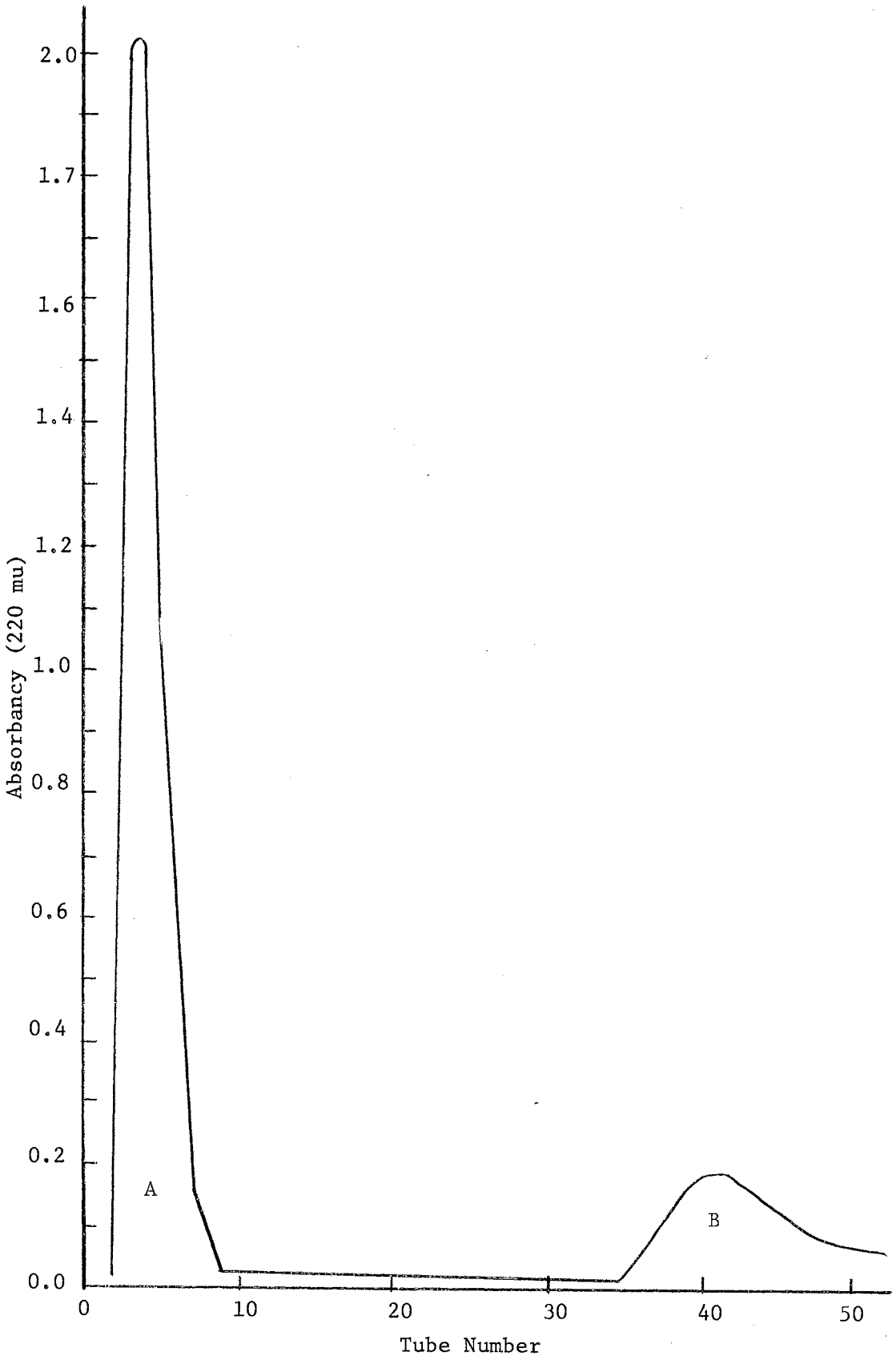
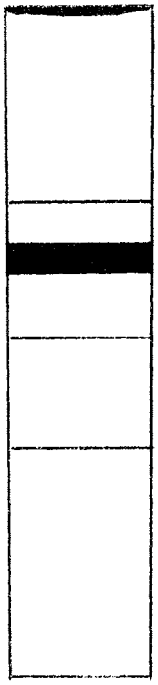

2.0
1.7
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Absorbancy (220 mu)
A
B
0
10
20
30
40
50
Tube Number

Figure 20. Comparison of protein profiles obtained from reaggregated membranes and gel filtration chromatography fractionation.

A. Reaggregated membrane obtained at pH 9.1 with limited Mg++.

B. Reaggregate obtained from Peak B in Figure 19.

A



B

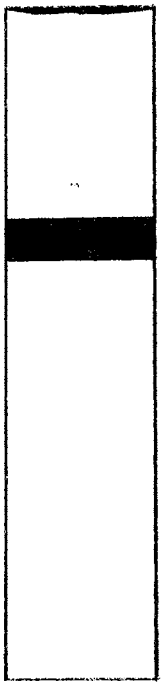


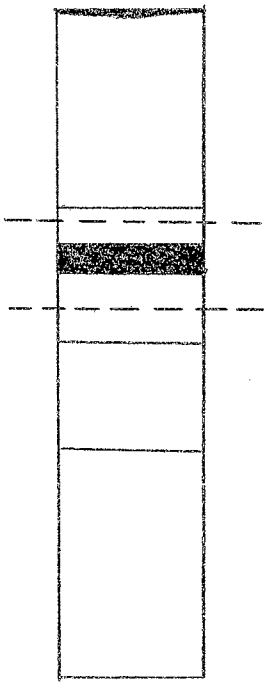
Figure 21. Protein profile from electrophoretic isolation of major protein species.

A. Protein profile of reaggregated membrane (pH 9.1 limited Mg++).

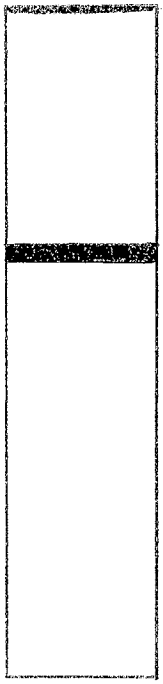
B. Protein profile of the sample obtained from the isolation procedure.

Dashed lines enclose the section of the gel which was removed.

A



B



CHAPTER IV

SUMMARY AND CONCLUSIONS

In summary, it can be concluded that the conditions for membrane reaggregation are essentially the same for Mycoplasma laidlawii, Micrococcus lysodeikticus, and Erwinia species. Reaggregation requires removal of the detergent used to solubilize the membrane and the presence of a divalent cation (Mg^{++}). Hydrophobic bonding appears to be the major chemical force involved in cell membrane aggregation, although a divalent cation is necessary for full stability and possibly, the final ordered form of the sheets.

The phospholipids that comprise a large percentage of the cell membrane do not appear to play an obligatory role in cell membrane structure because their extraction from reaggregated membrane sheets does not cause dissolution of the sheets. Further, membranes from which the lipids have been extracted can be disaggregated and subsequently reaggregated in the absence of phospholipids. These findings are in agreement with those published by Grula, et al. (1967).

In addition, we have observed that the number of proteins present in a reaggregated membrane preparation are dependent upon the concentration of Mg^{++} and pH of the dialyzing medium. Either high concentrations of Mg^{++} or low pH values in the dialyzing medium cause more proteins to appear in the reaggregated membrane preparations. These findings may indicate that many of the membrane associate proteins

are probably attached to the membrane by ionic bonding or possibly through the mediation of divalent cations.

The major chemical components of reaggregated membrane of Erwinia species are protein and phospholipid. Very small amounts of lipopolysaccharide (LPS) and cell wall lipoprotein (HPM) are present. It would appear that the conditions for reaggregation of the LPS and HPM differ from those used to permit aggregation of cell membrane subunits.

No 260 mu absorbing material could be detected in TCA extracts of reaggregated membrane. This finding indicates that little or no nucleic acids are associated with the reaggregated membrane.

The major structural protein of the membrane of Erwinia sp. can be purified to a large extent by reaggregation at pH 9.1 with limited Mg^{++}. This protein species has similar electrophoretic properties to what is thought to be the major structural protein present in the membrane of Micrococcus lysodeikticus (unpublished data). The molecular weight of the structural protein of Erwinia species cell membrane is estimated to be $35,000 \pm 5,000$.

Complete purification of this protein can be accomplished by preparative disc gel electrophoresis, although yields thus far have been disappointingly low. Sephadex gel filtration chromatography, when performed in the absence of SLS, seems to provide a method for isolation of sufficient quantities of purified structural protein to make further study of its nature possible.

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VITA 2

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