MICROCOCCUS LYSODEIKTICUS: MEMBRANE DISAGGREGATION AND REAGGREGATION

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DISAGGREGATION AND REAGGREGATION

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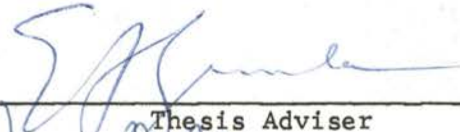

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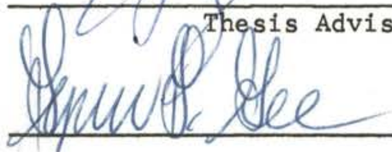

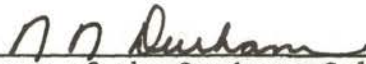

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CHAPTER I

INTRODUCTION

Weibull (1953b) observed that the enzyme, lysozyme, will hydrolyze and cause removal of the cell wall of Bacillus megaterium converting the rods into spherical protoplasts. The protoplasts are unstable in distilled water or isotonic salt solutions and will lyse unless protected under hypertonic conditions with sucrose or other suitable stabilizing substances. Lysis of protoplasts, by dilution with distilled water, will produce an empty shell or "ghost" (cell membrane). Thus, Weibull demonstrated unequivocally that a bacterial cell membrane exists and functions as an osmotic barrier. The techniques developed by Weibull have been successfully used to isolate bacterial cell membranes especially from those Gram-positive bacteria which are sensitive to lysozyme.

The most noted and perhaps the primary function of the bacterial cell membrane is its role as a selective permeability barrier. Weibull (1955) demonstrated the permeability capabilities of membranes by increasing the concentration of external medium and obtaining a decrease in protoplast volume and vice versa. This volume change due to osmotic pressure occurred because of a flow of low molecular weight compounds through the membrane. He also demonstrated that protoplasts which were suitably protected (hypertonic conditions) retained small molecules.

More recently the biological role of bacterial membranes has been expanded to include attachment sites for cytochromes, electron transport systems, and certain enzymes. Stanier (1954) proposed that the bacterial cytochrome system was a built-in part of the cell membrane and functioned in the manner similar to the mitochondria of higher organisms. Salton and Emitisham-ud-din (1965) verified that cytochromes were present in the membranes of Micrococcus lysodeikticus. Also, Weibull (1953a, 1955) found succinic dehydrogenase and DPNH oxidase in membranes of B. megaterium, strain M, along with pigmented material containing the cytochrome system. Strain KM of the same organism has lactic dehydrogenase activity in the "ghost" fraction (Storch and Wachsman, 1957). Mitchell and Moyle (1956) measured high malic and lactic dehydrogenase activity in membranes prepared from Staphylococcus aureus.

The bacterial membrane has been designated as the location for synthesis of exocellular enzymes. Lampen (1965) has reviewed the role of the membrane as an attachment site for polysomes which form the enzyme protein. Kushner and Pollack (1961) suggested penicillinase was formed on the membrane and released into the external environment.

The bacterial cell membrane is also involved in the synthesis of cell wall precursors. An example exists in B. megaterium where the peptide component of the cell wall is synthesized at the membrane surface (Brooks, Crathorn, and Hunter, 1959). Deitrich, Matsuhashi, and Strominger (1965) have also noted the importance of the membrane in cell wall synthesis after isolation of a phospholipid-cell wall precursor from S. aureus.

Several investigators have observed growth and division in Gram-positive protoplasts and Gram-negative spheroplasts (McQuillen, 1960; Martin, 1964). L-Forms of Proteus mirabilis, devoid of any cell wall material, can divide indefinitely (Martin, 1964). Grula and Grula (1964) suggested that the cell membrane rather than the cell wall initiates cell division in a species of Erwinia.

As the biological role of the bacterial cytoplasmic membrane expanded, its composition became of interest. The membrane in Gram-positive bacteria is a complex of lipoprotein containing the majority of cellular lipids. The lipid portion is composed mainly of phospholipids; in some bacteria a small amount of carotenoid may also be present. Gilby, Few, and McQuillen (1958) have reported that membrane material from M. lysodeikticus makes up 8.6% of the cell dry weight and is composed of 28% lipid, 50% protein, and 15 to 20% carbohydrate. The lipid fraction is 80% phospholipid (polyphosphatidic acid); a portion of the remaining lipid was found to contain carotenoid responsible for the yellow pigment of the organism. Mannose is the only carbohydrate present. Trace amounts of nucleic acids were also reported. The membrane composition of other organisms has been studied and varies little from that above. Some variations in types of phospholipids have been noted such as the presence or absence of the nitrogen-containing phospholipids ethanolamine, serine, and choline.

It has been postulated that the phospholipid portion of the membrane is so oriented that the hydrophobic fatty acid side chains mesh inwardly and maintain their position through van der Waals forces (Robertson, 1959). The polar portions of the phospholipids are oriented externally and bind protein ionically on the external and

internal surfaces (Green and Tisdale, 1961). Therefore, the overall structure is thought to be three layered; the bimolecular leaflet of phospholipid with two outer protein layers. This theory of a "unit membrane" structure (sandwich) has been proposed for mitochondria, mammalian cells, and bacterial cells. Data supporting the theory have been obtained from electron microscopic and X-ray diffraction studies (Robertson, 1959).

Many theories on the structural makeup and function of biological membranes have developed from work with fatty acid monolayers. Polyvalent ions act to strengthen the monolayers and form films resembling membranes (Danielli and Davson, 1935). It is believed that these ions either reduce charge on the fatty acids allowing close association for van der Waals forces or bond with the ionic groups of the fatty acids forming a lattice structure (Danielli and Davson, 1935; Rogeness and Abood, 1964). In either case the ions interact to form a hydrophobic area. Similar reactions involving ions could operate during *in vivo* synthesis of the cytoplasmic membrane.

Green and Hechter (1965) have recently taken issue with the "unit membrane" theory of Danielli and Davson. They reason that if phospholipid is present between two layers of protein, the phospholipids should be responsible for holding the protein layers apart; removal of the phospholipids should cause collapse of the sandwich. Because no collapse occurs when phospholipids are removed (acetone extraction), it is their contention that phospholipids do not occupy the inner area of the membrane as proposed by Danielli and Davson.

The mitochondrial membrane has been very useful as a functioning system to aid in determining the structure and function of biological

membranes. The functional unit of the mitochondrial membrane has been referred to by Green and Hechter (1965) as the elementary particle. This lipoprotein unit is made up of all the functioning parts of the mitochondrion such as the electron transport system, ATP synthetases, translocases involved in ion translocation, and structural protein. Several workers have been able to dissociate several functional parts of the elementary particle, isolate them, and reconstitute them without loss of function (Green and Hechter, 1965; King, 1962; Hatefi, Haavik, Fowler, and Griffiths, 1962). Reconstitution of the mitochondrial subunits is primarily through hydrophobic bonding of nonpolar protein regions and lipids (Green et. al., 1961).

Similar work has been performed using the PPLO organism, *Mycoplasma laidlawii*. This organism has no cell wall and the cytoplasmic membrane is the limiting boundary between the cell and its environment. The membrane of this PPLO is very similar to the bacterial membrane in that it is composed of lipoprotein units (Razin, 1963). Electron microscopy reveals the typical three layered structure "unit membrane" as noted by others in biological membranes. Razin, Morowitz, and Terry (1965) have disaggregated the PPLO membrane into a uniform sized subunit using sodium lauryl sulfate. During dialysis in the presence of magnesium ions, the subunits reaggregate into a structure resembling the original. Electron microscopy of the reaggregated material has revealed a three-layered structure, and DPNH oxidase activity is also present. These workers postulate that ionic and apolar bonding are responsible for the reaggregation. These types of reaggregation may be considered as fabrication of supra-molecular units and occur without mediation of enzymes. Apparently the information necessary for reaggregation is

present within the subunit molecular complexes.

It is believed that several antibiotics and antimicrobial agents exert their effects at the level of the bacterial cell membrane. For example, growing cells in the presence of penicillin will cause lysis or, if the cells are osmotically protected, cause formation of spheroplasts or protoplasts. Grula and Grula (1964) reported that penicillin inhibited growth and division in a species of Erwinia. Pantoyl lactone or hypertonic conditions will reverse division inhibition and in several cases stimulate or allow growth. The effect of hypertonic conditions in particular on division inhibition by penicillin would suggest membrane damage by the antibiotic. Evidence for a site of activity of penicillin was provided by Cooper (1954, 1956) who proposed a penicillin-binding component (PBC) composed of phospholipid, other lipid, and cell wall material. Duerksen (1964) supported Cooper by demonstrating that the receptor site for penicillin binding in Bacillus cereus is lipoprotein or a compound closely associated with lipoprotein. He reported that the receptor site is released during protoplast formation and is not present either in isolated cell walls or membranes. Therefore, Duerksen has suggested that the PBC exists in a position between the wall and membrane. Shockman and Lampen (1962) found no inhibition of protoplast growth in the presence of penicillin and, therefore, stated that the site of action of penicillin was solely on cell wall synthesis. It should be pointed out that the protoplasts were osmotically protected during their experiments and such protection could mask possible subtle disruptive effects by penicillin on the membrane.

The antibiotic cycloserine (oxamycin) has been compared to penicillin since it can cause lysis of sensitive growing cells (Shockman and Lampen, 1962). This effect is also attributed to inhibition of cell wall synthesis. Grula and Grula (1965) reported that mucopeptide content of cycloserine-treated Erwinia sp. was reduced by 40% concomitant with inhibition of growth and cell division. These investigators proposed secondary membrane damage to the cell both by cycloserine and penicillin since hypertonic conditions overcame growth and division inhibition.

The bactericidal effects of mitomycin c such as depolymerization of DNA by activation of a DNase or intercalation into complementary strands of DNA have been reported (Reich, Shatkin, and Tatum, 1961). However, Grula and Grula (1962) using Erwinia sp. reported that mitomycin c caused leakage and inhibition of mucopeptide synthesis. The effects of mitomycin c on leakage, growth, and division were greatly alleviated in hypertonic media again suggesting membrane involvement in the division process.

Several workers have reported that vancomycin affects the bacterial cell membrane. Grula and Grula (1964) also reported inhibition of growth and division in Erwinia sp. by vancomycin. Again, as in the case with penicillin, cycloserine, and mitomycin c, hypertonic conditions restore division activity without a concomitant restoration of mucopeptide formation. Shockman and Lampen (1962) suggested that vancomycin affected the membrane since growth inhibition of protoplasts occurred in the presence of the antibiotic. Hancock and Fitz-James (1964) demonstrated an efflux of potassium ions from cells treated with vancomycin. This action is also reversed under hypertonic

conditions indicating damage to the membrane. Recently Chatterjee and Perkins (1966) have shown that vancomycin can adsorb to cell wall precursors located in or on the cell membrane. Durham and Best (1965) demonstrated that vancomycin will attach to the cell wall of Bacillus subtilis.

A portion of the lipid content of bacterial membranes consists of unsaturated carotenoids. These compounds are responsible for the pigmentation of M. lysodeikticus and possess characteristic absorption peaks useful in following membrane isolation and behavior. Diphenylamine can alter the carotenoids during growth to the extent that no pigment is observed. Cho, Corpe, and Salton (1964) have reported the loss of carotenoid absorption patterns when the cells are grown in the presence of diphenylamine. Goodwin (1954) postulated that diphenylamine stimulated the saturation of carotenoid by inhibiting dehydrogenation, thus, leading to a loss in color.

The polyamine compounds spermine, spermidine, and putrescine have been reported to affect membrane stability in addition to their ability to react with nuclear and ribosomal units. Mills and Dubin (1966) have reported that spermine increases cell permeability and causes cell lysis but only when the cells are initially resuspended in a spermine-free medium. It would seem that spermine inhibits protein synthesis while prolonging cell life by stabilizing the membrane. Tabor and Tabor (1966) support the theory of membrane interaction with spermine by demonstrating two systems for uptake of spermine in Escherichia coli. One system requires no energy and appears to be simple adsorption to the cell surface since the spermine can easily be removed by washing; the other system is energy dependent and the spermine cannot

be washed from the cells. Razin, Morowitz, and Terry (1966) were able to reaggregate membrane subunits from Myco. laidlawii using spermine in place of magnesium. Therefore, it is clear that polyamine compounds can exert biological activity at the level of the cell membrane.

Since reaggregation of membrane subunits is possible under controlled conditions, we wished to develop a system that would allow study of the reaggregation phenomenon. Aside from the need to study and more fully understand this form of synthesis of supramacromolecular units, it was hoped that information gained might be extrapolated to the cell membrane system of Erwinia sp. and cell division activity. M. lysodeikticus is an ideal organism for this type of study since clean membranes can easily be obtained following digestion of the cell wall with lysozyme and osmotic lysis of the resulting protoplasts. Yellow pigments present in the cell membrane of M. lysodeikticus make it possible to follow membrane associated phenomena with a degree of confidence not possible in a non-pigmented bacterial species such as Erwinia.

CHAPTER II

MATERIALS AND METHODS

Test Organism

The organism used in this study was the Purdue University strain of *Micrococcus lysodeikticus* believed to be a transplant from the original strain isolated by Fleming. Grula (1962) has compared the organism with five other strains of *M. lysodeikticus* and fully described biochemical, nutritional, and antibiotic sensitivity characteristics. The organism is described in the sixth edition of Bergey's Manual only as a yellow micrococcus easily lysed by lysozyme. It is not listed in the seventh edition of Bergey's Manual. *M. lysodeikticus* is easily converted to protoplasts using lysozyme.

Stock cultures of the organism were maintained on nutrient agar slants incubated at 30° C and stored at 4° C. Twenty-four hour old cells were used as a source of inoculum for all experiments. The streak plate method was occasionally employed to aid in detecting possible contamination of the culture.

Medium and Growth

A modification of the defined medium of Grula, Luk, and Chu (1961) and Grula (1962) was employed. This medium is composed of the following compounds per 100 ml: biotin (50 ug), inosine (6.0 mg), L-glutamic acid (358 mg), L-phenylalanine (40 mg), L-tyrosine (30 mg), NH_4Cl (100 mg), Na_2HPO_4 (200 mg), and $MgSO_4 \cdot 7H_2O$ (2.0 mg). The pH of the

complete medium was adjusted to pH 7.6 - 7.8 using solid KOH. Sterilization of the medium was accomplished by autoclaving 12 minutes at 10 pounds pressure. A solution of $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$ was autoclaved separately and the resulting red precipitate was allowed to settle before aseptic additions were made to the medium. A final concentration of approximately 25 ug ferrous ammonium sulfate per 100 mls medium was used.

The medium was inoculated with cells grown on nutrient agar slants at 37° C. After washing once in 0.85 percent sterile saline, the cells were adjusted to an optical density of approximately 0.30 (Coleman Jr. Spectrophotometer). One drop of this suspension was used per 5.0 ml of medium in culture tubes. For larger cell yields, 100 ml aliquots of the defined medium were poured into 250 ml Erlenmeyer flasks and 1.0 ml of inoculum was added. Incubation was accomplished at 30° C using a rotary shaker for approximately 30 hours. At this time, cell cultures are in the late logarithmic phase of growth and have an optical density of 0.28 - 0.32 (Coleman Jr. Spectophotometer 540 mu).

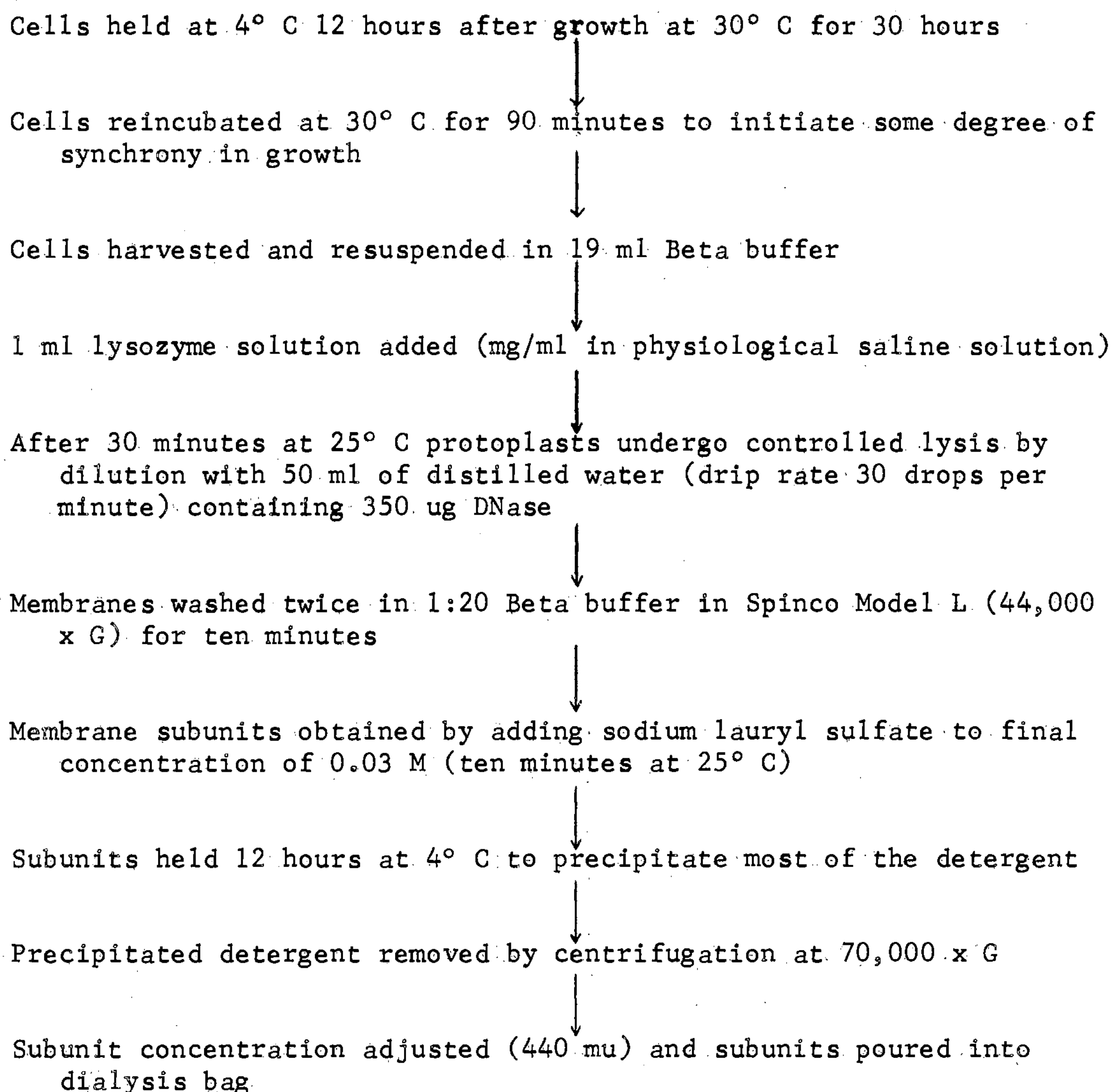
Isolation of the Bacterial Cell Membranes and Preparation of Subunits

Cells were placed at 4° C overnight after a 30 hour growth period and reincubated at 30° C for 90 minutes the following day. This step initiated some degree of synchrony to cell growth and allowed for greater and more uniform lysozyme sensitivity. The cells were then harvested by centrifugation and resuspended in the Beta buffer of Razin, Morowitz, and Terry (1965) (0.156 M NaCl, 0.05 M Tris, and 0.01 M 2-mercaptoethanol adjusted to pH 7.4 using HCl). Cells from 500 ml medium were resuspended in 19 ml of the buffer, 1.0 ml of lysozyme was

added (mg per ml in physiological saline) and the suspension incubated 30 minutes at 25° C to obtain protoplasts. The protoplasts were slowly lysed by dilution of the buffer-cell mixture with 50 ml of distilled water. This procedure was performed at 4° C by dripping the water at a rate of approximately 30 drops per minute. This method reduced the tendency for explosive plasmoptysis and therefore a minimum amount of damage to the membranes resulted. In order to decrease viscosity of the lysed protoplast suspensions, deoxyribonuclease was added to the diluting water solution to allow for a final concentration of 5 ug per ml in the protoplast suspension. Deoxyribonuclease was allowed to react for 30 minutes at 4° C prior to further processing of the membranes. The membranes were washed twice by centrifugation at 44,000 x G for 30 minutes (Spinco Model L) in 1:20 Beta buffer then resuspended in 9.0 ml of the buffer. To prevent foaming during formation of membrane subunits, the membranes were suspended in the buffer prior to addition of 1.0 ml sodium lauryl sulfate (SLS) at 0.3 M giving a final detergent concentration of 0.03 M. After ten minutes at 25° C the membrane subunits were placed at 4° C overnight. At this temperature, most of the SLS precipitates and can be removed by centrifugation at 70,000 x G for 30 minutes. The membrane subunits remain suspended in the supernatant due to their small size. A flow diagram for these manipulations is presented in Figure 1.

Figure 1

FLOW CHART FOR PREPARATION OF CELL MEMBRANES AND MEMBRANE SUBUNITS FROM M. LYSODEIKTICUS



Dialyzing Technique

The membrane subunit concentration was adjusted by dilution with 1:20 Beta buffer until an optical density between 0.09 and 0.15 was obtained at 440 mu (Coleman Jr. Spectrophotometer). This wavelength was selected since it is near to an absorption maximum for carotenoid found in membranes in M. lysodeikticus. The subunit suspension was placed in dialysis tubing and dialyzed using a volume ratio (inside-outside) of 1:100 (Razin, Morowitz, and Terry, 1965). The dialyzing solution contained 0.01 M $MgCl_2$ in 1:20 Beta buffer and was changed after the first twenty-four hours. Dialysis was continued for a period of four to seven days until a yellow turbidity (fluff) could be observed in the bag. At this time, the reaggregated membranes were centrifuged at 70,000 x G and obtained as a yellow pellet.

Quantitation of Reaggregation and Absorption Spectra

The amount of membrane reaggregated in experimental situations was compared to a control by reading the optical density of the supernatant and resuspended membrane pellet. These readings were taken at 440 mu for carotenoid absorption and at 640 mu for optical density of the suspension (Coleman Jr. Spectrophotometer). Further detection of membranes was accomplished by obtaining an absorption spectrum of the resuspended pellet material between 400 and 500 mu (5 mu intervals) using either a DU or a Cary spectrophotometer. These membranes were suspended in glycerine and 1:20 Beta buffer (1-1) to reduce opacity of the suspensions (Stanier, Gunsalus, and Gunsalus, 1953).

Agents Affecting Membrane Reaggregation

Various agents were added to the dialyzing solution to determine their affect on the reaggregating capability of membranes. A solution of vancomycin was prepared at 4° C and added to prechilled dialyzing solution to a final concentration of 175 mg per liter. Diphenylamine, insoluble in water, was suspended in ethanol and then added to the dialyzing system to a final concentration of 25 mg per liter. The polyamine compounds spermine, $H_2N(CH_2)_3NH(CH_2)_3NH_2$, spermidine, $H_2N(CH_2)_4NH(CH_2)_3NH_2$, and putrescine $H_2N(CH_2)_4NH_2$ were solubilized in water and the pH adjusted to neutrality using HCl. Final concentration of these compounds was 0.003 M during dialysis. Pantoyl lactone (0.01 M final concentration) was used for dialysis after adjustment to a pH of 7.4. Penicillin was added to 100, 1000, and 5000 units per ml during dialysis. In all instances these compounds were tested in the presence and absence of magnesium in the dialyzing system.

Membrane subunits were also dialyzed using various cations to replace magnesium at the standard final concentration of 0.01 M. The divalent cations zinc, calcium, and manganese were used; for trivalent cations iron and aluminum were employed. All metal cations were tested as their chloride salts.

Phospholipid Isolation

Cells grown in the defined medium were harvested and resuspended in 20 ml methanol to separate lipid-protein complexes. An atmosphere of nitrogen was added to the suspension to prevent air oxidation. After heating in a 55° C water bath for 30 minutes, two volumes of chloroform were added. Nitrogen was flushed through the system which was then

held at 25° C for 12 hours. The chloroform-methanol solution was filtered through a millipore filter to remove particulate cell material. The filtrate was then washed twice with equal volumes of 2.0 M KCl and once with an equal volume of distilled water; the aqueous layer was removed each time by aspiration. The resulting chloroform fraction containing phospholipids was concentrated by evaporation under vacuum.

Phospholipid Chromatography

Phospholipids were identified using thin-layer chromatography as described by Stahl (1960). Glass plates, 20 x 20 cm square, were layered with Silica Gel G (Merck) 250 microns in thickness and dried at 25° C. The plates were then activated at 100° C one hour and stored under vacuum. Thin-layer chromatograms were developed (ascending) in chloroform-methanol-water (65:25:4) after a one hour equilibration period. Substances containing lipid were identified by spraying with rhodamine (0.001 percent) and observing under ultraviolet light (Luzzati and Husson, 1962). Phospholipids containing amino groups were detected using 0.5% ninhydrin in 95% acetone and heating at 105° C for three to five minutes. Phosphate-containing lipids were detected using the molybdate spray of Hanes and Isherwood (1949). Choline-containing phospholipids were identified using the Dragendorf reagent spray of Wagner, Hornhammer, and Wolff (1961).

Amino Acid Chromatography

Samples for amino acid analysis were hydrolyzed in 6.0 N HCl 18 hours at 105° C _in vacuo_. The hydrolyzate was dried by evaporation under a warm flow of air (hair dryer) and washed twice in distilled

water utilizing the same method. After resuspension in a small volume of water, the hydrolyzate was spotted on Whatman number 1 filter paper for chromatography. Chromatograms were developed ascending in two directions using the solvent systems of Redfield (1953). The thin-layer chromatographic system of Heathcote and Jones (1965) was also employed for amino acid separation and identification. Amino acids were detected by spraying with 0.5 percent ninhydrin in acetone water (95-5 v/v) and heating at 65° C for 30 minutes.

Amino Sugar Chromatography

Material thought to contain amino sugars was hydrolyzed *in vacuo* four hours in 4.0 N HCl at 105° C. The hydrolyzate was spotted on Whatman number 1 filter paper and developed in the two-dimensional system of Redfield (1953). The amino sugars were detected by the method of Morgan and Elson as described by Partridge (1948) and appeared as reddish-brown spots. Analysis for amino sugars (glucosamine and muramic acid) was also performed by Dr. Mary Grula utilizing a more sensitive test tube colorimetric technique (Rondle and Morgan, 1955).

Preparation of Membrane Subunits by Sonic Disruption

Isolated cell membranes were reduced to their subunit form by sonic disruption using the Branson Sonifier unit. Sonic disruption was accomplished utilizing 15 second bursts at maximum output followed by two minute intervals for cooling in an ice bath. This step was repeated four to eight times to insure subunit formation. Remaining whole membranes were removed by centrifugation at 70,000 x G (Spinco Model L). Optical density readings at 440 mu revealed the presence of membrane

subunits in the supernatant.

Ultracentrifugation

Centrifugation of membrane subunits was accomplished using the Spinco Model E analytical ultracentrifuge. Subunits prepared by detergent action or sonic disruption were centrifuged at 59,780 rpm for one hour at 20° C. At ten minute intervals photographs recorded the sedimentation peaks. From these peaks it was possible to calculate uncorrected sedimentation constants. These studies were accomplished in the Department of Chemistry and were supervised by Dr. George Gorin.

Electron Microscopy

Whole cell membranes and reaggregated membranes were fixed by the addition of 6.25 percent glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) to the pellets for two hours at 4° C, rinsed in 0.1 M sodium cacodylate buffer, cut into small blocks, and post-fixed for one hour using one percent osmium tetroxide in Michaelis acetate-veronal buffer at pH 6.1 (Razin, Morowitz, and Terry, 1965).

The material was rinsed with distilled water and dehydrated through a series of ethyl alcohol rinses (25%, 50%, 75%) for fifteen minutes in each grade followed by three washes of fifteen minutes each in 95 and 100 percent ethyl alcohol. The alcohol was removed by three fifteen minute washes using propylene oxide. The material was embedded in Araldite (Robson, 1964) and sectioned on a Sorvall MT-2 Ultramicrotome. Sections were picked up on uncoated 400 mesh copper grids, stained fifteen minutes in a saturated solution of uranyl acetate in 50 percent ethanol, washed in distilled water, and dried on filter

paper (Razin, Morowitz, and Terry, 1965). Specimens were examined in an RCA EMU-3G microscope.

In addition, small drops of whole membranes and reaggregated membrane sheets were spotted on 400 mesh copper grids, which were coated with collodion and stabilized with a thin carbon film. The drops of membrane material were allowed to sit for 30 seconds, blotted with filter paper, and a small drop of 2% uranyl acetate or 2% phosphotungstic acid was then placed over the membrane material. After 30 seconds the uranyl acetate was drawn off using filter paper, and the grid allowed to dry. Specimens were then examined in an RCA EMU-3G microscope at an accelerating voltage of 100 Kv.

CHAPTER III

RESULTS AND DISCUSSION

Conditions for Membrane Reaggregation

Reaggregation of membrane subunits from M. lysodeikticus was initially accomplished using the system of Razin, Morowitz, and Terry (1966). Since these investigators used membrane subunits from Mycoplasma laidlawii, we wanted to determine if the same optimum conditions existed for our organism. Beta buffer was tested first at 1 in 5, 1 in 10, and 1 in 20 dilutions with equal concentrations of magnesium and membrane subunits for each situation. After six days of dialysis at 4° C, spectrophotometric data (Table I) indicated that lower concentrations of buffer yielded greater amounts of reaggregation. Therefore, the 1 in 20 buffer dilution used by Razin, Morowitz, and Terry (1966) was also selected for our reaggregation system.

TABLE I

DETERMINATION OF OPTIMUM BUFFER CONCENTRATIONS FOR MEMBRANE REAGGREGATION

Buffer Dilution	O.D. at 440 mu
1 in 5	.66
1 in 10	.75
1 in 20	.96

The optimal magnesium concentration for dialysis was next determined by reaggregating membrane subunits using 0.01 M and 0.005 M magnesium. A control containing no magnesium was also run to determine if a divalent cation was necessary for membrane reaggregation. The subunits were dialyzed six days at 4° C. Results of this experiment are listed in Table II.

TABLE II

DETERMINATION OF Mg^{++} CONCENTRATION FOR MEMBRANE REAGGREGATION

Situations Studied	O.D. at 440 mu	O.D. at 640 mu
Reaggregate		
0.01 M Mg^{++}	.64	.17
0.005 M Mg^{++}	.60	.15
No Mg^{++}	.08	.02
Supernatant		
0.01 M Mg^{++}	.04	0.00
0.005 M Mg^{++}	.07	0.00
No Mg^{++}	.09	0.00

Optical density readings at 440 mu revealed that large amounts of carotenoid pigment were present in reaggregated material formed in the presence of 0.01 or 0.005 M Mg^{++} ion. This was substantiated by readings made at 640 mu which demonstrated that a far greater amount of particulate matter was obtained when Mg^{++} ion was present during dialysis. A small amount of reaggregation might have occurred in the absence of magnesium, however, it is evident that presence of the cation resulted in a many fold increase in reaggregation. Therefore, although both concentrations of Mg^{++} ion allowed good reaggregation the

higher magnesium concentration (0.01 M) was selected for use in all future reaggregation experiments. This is the same concentration used by Razin and his co-workers.

The effect of temperature on dialysis was resolved by reaggregating membrane subunits at 4°, 20°, 30°, and 37° C. After five days, large amounts of reaggregated material were obtained; optical density data are presented in Table III.

TABLE III

EFFECT OF TEMPERATURE ON MEMBRANE REAGGREGATION

Temperature	Supernatant 440 mu	Supernatant 640 mu	Reaggregate 440 mu	Reaggregate 640 mu
4°	0.04	0.0	0.46	0.17
20°	0.02	0.0	0.92	0.41
30°	0.0	0.0	1.0	0.43
37°	0.0	0.0	1.09	0.60

The fact that higher temperatures increase membrane reaggregation is probably due to increased motion in the system and, therefore, greater probability of contact between subunits. It was decided to use 4° C as the standard temperature for reaggregation since higher temperatures could allow more rapid degradation of subunits by bacterial contaminants or loss of enzyme activity (denaturation) within the protein-containing subunits.

Membrane subunits were also reaggregated in buffer with pH adjustments to 6.4, 7.4, and 8.4. These results are shown in Table IV. At

pH 6.4 the excess hydrogen ion converted residual sodium lauryl sulfate to the acid form (lauric acid) resulting in a white precipitate in the dialysis bag. No legitimate optical density readings could be obtained; however, after centrifugation, yellow material was observed in the predominantly white pellet indicating that some reaggregation did occur at the lower pH level. The highest amount of membrane reaggregation was observed at pH 7.4 (dialysis was for four days at 4° C).

TABLE IV

EFFECT OF pH ON MEMBRANE REAGGREGATION

pH of Buffer	440 mu	640 mu
6.4	precipitate	precipitate
7.4	.66	.22
8.4	.43	.12

In other experiments, mercaptoethanol and sodium chloride were removed from the dialyzing solution. After dialyzing four days at 4° C, the reaggregated membrane material was sedimented and resuspended for optical density readings (Table V).

Mercaptoethanol was found not to be necessary for membrane reaggregation. Also, removal of sodium chloride enhanced reaggregation. This latter finding indicates that optimal reaggregation requires a low ionic strength menstruum.

TABLE V

BUFFER REQUIREMENTS FOR MEMBRANE REAGGREGATION

Situation	440 mu	640 mu
No Mercaptoethanol	0.48	0.19
Control	0.47	0.22
No Sodium Chloride	0.43	0.10
Control	0.37	0.08

To confirm the need for a low ionic strength menstruum, reaggregation was also attempted in the presence of 2 M sodium chloride. At this level of sodium chloride, a heavy white precipitate which is soluble in buffer at room temperature forms in the dialysis bag and very little reaggregation is present.

In summary, our studies reveal that optimal conditions for reaggregation of membrane subunits from M. lysodeikticus differ from those reported for Myco. laidlawii by Razin and co-workers in that our system did not require sodium chloride or a reducing agent. Concentration of buffer, Mg^{++} ions, and pH appear to be very similar in both systems. No comparison can be made regarding temperature effects since Razin and co-workers did not report data relative to this parameter.

Comparison of Chemical Components of Whole and Reaggregated Membranes

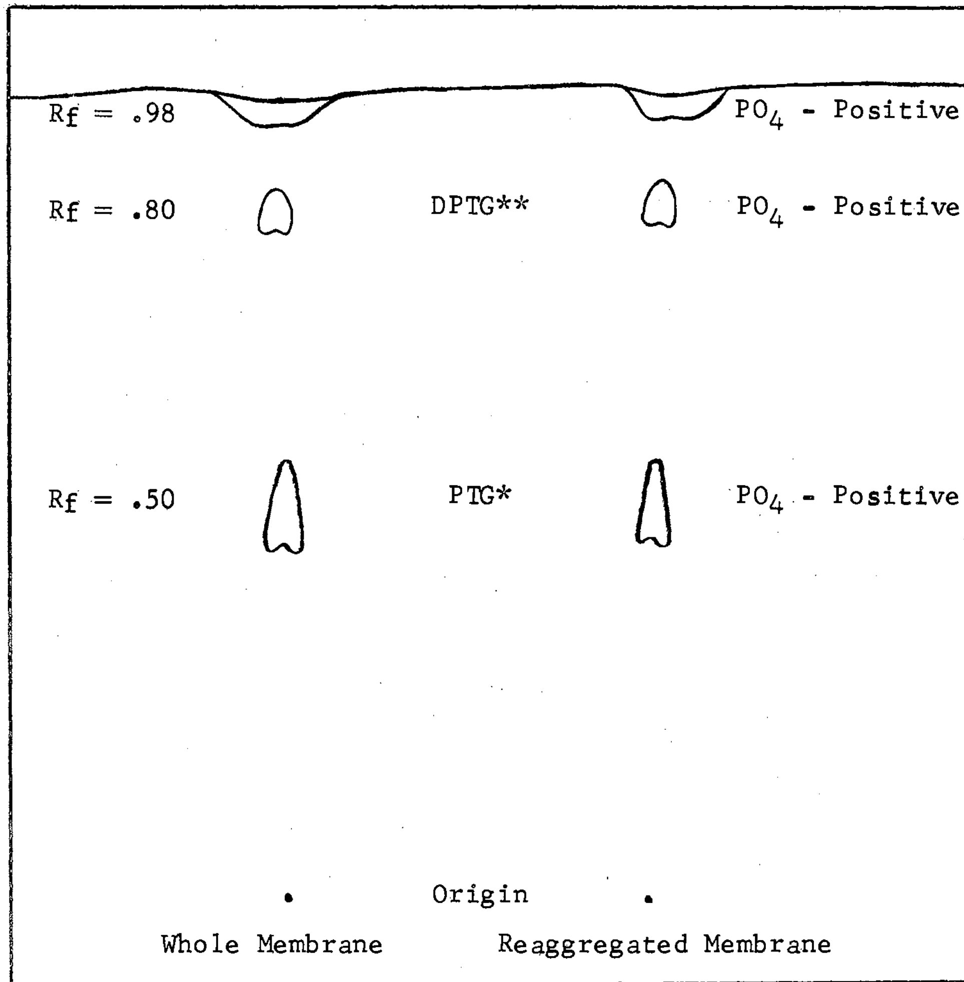
Using the yellow pellet obtained after centrifugation of the reaggregated fluff observed in dialysis bags, chemical analyses were performed for comparison to whole membranes. Amino acids, phospho-

lipids, and carbohydrates of whole and reaggregated membranes were found to be qualitatively the same.

The amino sugars, glucosamine and muramic acid (present in the cell wall), were found using paper chromatography, as contaminating entities in whole membrane preparations. We were unable to determine precisely, using paper chromatographic procedures, if these amino sugars were also present in reaggregates. Therefore, a more sensitive colorimetric technique performed by Dr. Mary Grula, was used to demonstrate that reaggregated membrane contained no glucosamine. A possible trace of muramic acid may have been present, however, the color was atypical. We have concluded that although some wall material remains associated with the cell membranes during the purification procedures, this wall material most probably does not become incorporated into the reaggregated membrane sheets.

Plasma or cytoplasmic membrane from *M. lysodeikticus* contains mannose as the only carbohydrate (Gilby, McQuillen, and Few, 1958). Hydrolysis of whole membrane preparations revealed one major carbohydrate-reacting material. This material migrated with mannose when co-chromatographed with this compound. Reaggregated membrane contained only one sugar which also migrated with mannose.

Phospholipids were also isolated from both whole and reaggregated membranes and chromatographed using thin-layer silica gel plates. When rhodamine was used for detection, nearly identical patterns were obtained for both membrane preparations (Figure 2). Three compounds were observed with Rf values at 0.50, 0.80, and 0.98; phosphate was present in all of these compounds. None of the phospholipid preparations contained ninhydrin-positive or choline-containing material.



**DPTG = Diphosphatidylglycerol

*PTG = Phosphatidylglycerol

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

Figure 2. Phospholipid Patterns of Whole and Reaggregated Membranes Obtained by Thin-Layer Chromatography

Although the phospholipids in our preparations have not been defined by rigid criteria we have, on the basis of published Rf values, position of spots, and non-reactivity with specific spray reagents concluded that phosphatidylglycerol is the major phospholipid (McIlwain and Ballou, 1966). Unfortunately, standards of this compound cannot be obtained for direct comparison.

Gilby, McQuillen, and Few (1958) postulated that the phospholipids of M. lysodeikticus exist as polyphosphatidic acid since after mild hydrolysis (methanolic KOH), compounds such as choline, ethanolamine, serine, or glycerol could not be detected. However, when more harsh hydrolysis methods were employed (6.0 N HCl) glycerophosphoric acid was obtained suggesting that phosphatidylglycerol was present. MacFarlane (1962) confirmed that phosphatidylglycerol was the major phospholipid present in the cell membrane of M. lysodeikticus.

Most of the naturally occurring amino acids were found to be present in protein hydrolyzates of whole membranes using the paper chromatography system of Redfield (1953) and the thin-layer technique of Heathcote and Jones (1965). Reaggregated membranes were hydrolyzed and, using the same chromatographic systems, found to contain the same amino acids. Data are presented in Table VI.

Physical Properties of Whole, Reaggregated, and Dissociated Membranes

Membranes from M. lysodeikticus were monitored throughout isolation and reaggregation by observation and/or measurement of their yellow carotenoid pigments which have absorption maxima at 417, 445, and 475 mu (Figure 3). The peaks at 445 and 417 mu were usually the highest and these would vary in amount depending on the preparation under

TABLE VI

AMINO ACIDS PRESENT IN WHOLE AND REAGGREGATED MEMBRANES*

Leucine	Tyrosine
Isoleucine	Alanine
Phenylalanine	Glutamic Acid
Threonine	Aspartic Acid
Valine	Serine
Lysine	Glycine
Methionine	Arginine
Proline	Histidine

* Cysteine and tryptophan destroyed during hydrolysis (6 N HCl for 18 Hr at 105° C).

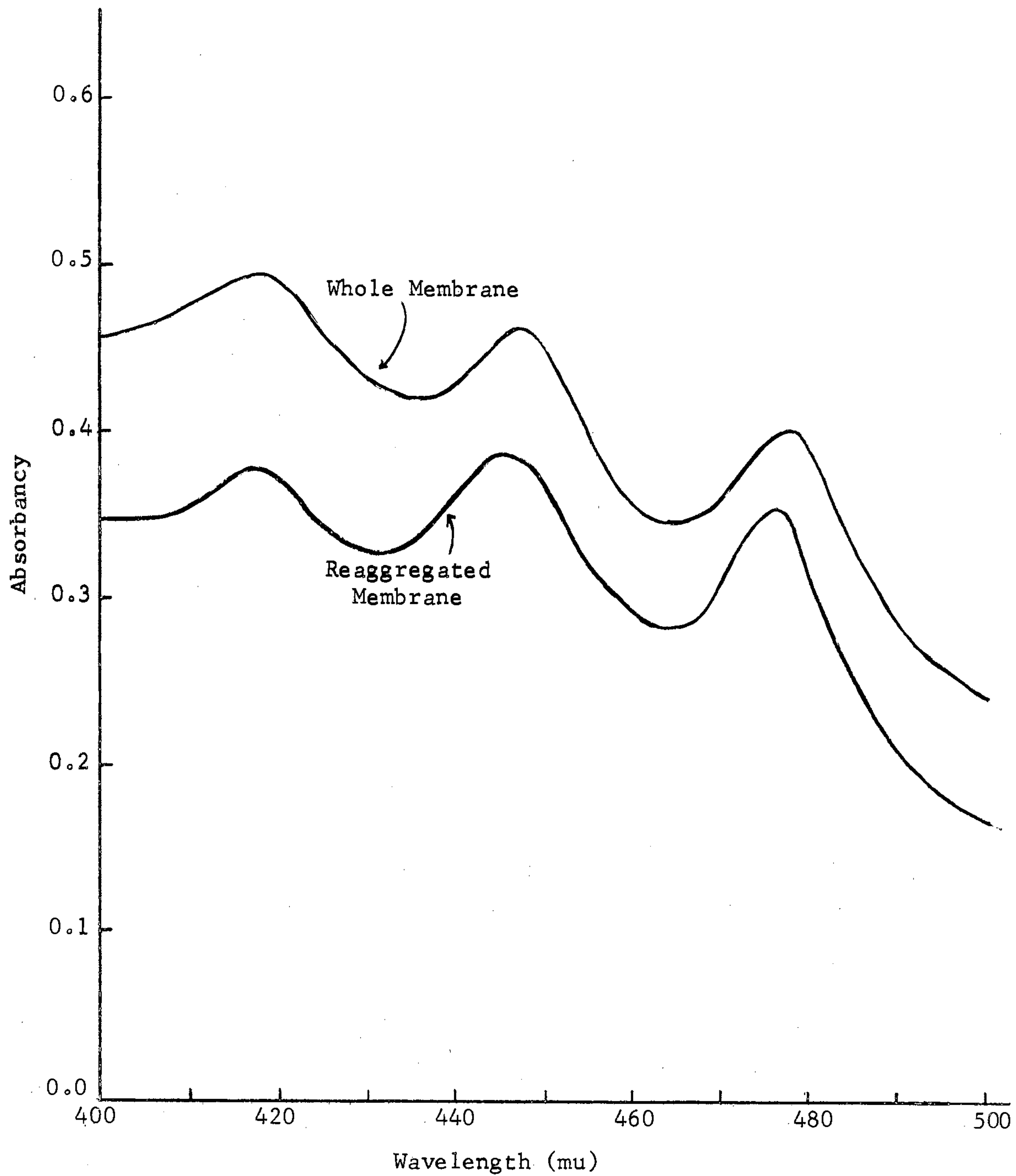


Figure 3. Absorption Spectra of Whole and Reaggregated Membranes

observation. Relative amounts of membrane could be determined by taking optical density readings at 440 mu. The data in Figure 3 allow the conclusion that reaggregated membrane sheets possess the same carotenoid pigments as exist in the whole membrane.

The detergent, sodium lauryl sulfate (SLS), when added to a whole membrane preparation changes it from an opaque yellow suspension to a yellow but clear "solution." After centrifugation of the SLS-treated membrane suspension, (70,000 x G for 30 minutes), no pellet can be obtained indicating that the membrane has been reduced to a very small size. Ultracentrifugation studies were undertaken to determine if the membranes had been reduced to various sized particles or existed as a uniform subunit. Figure 4 shows sedimentation patterns of membrane subunits taken at ten minute intervals in the Model E ultracentrifuge. The appearance of only one symmetrical peak suggests that detergent-treated membrane produces a single subunit of uniform size. An average sedimentation constant, uncorrected for viscosity and specific gravity, was calculated to be 3.3 Svedberg units.

Electron micrographs of whole membrane (Figure 6) revealed the ghost cell appearance first observed by Weibull (1953). These micrographs also show that physical damage to membranes during controlled lysis and two washing steps is not completely disruptive. Whole membranes, thin-sectioned and stained with uranyl acetate, show the typical three-layered unit membrane structure (Figure 8). Disaggregation of membrane by SLS completely destroys their structure; this was confirmed using the electron microscope.

The overall structure of reaggregated membrane is shown in Figure 7 and appears as sheet-like material possessing various shapes and sizes.

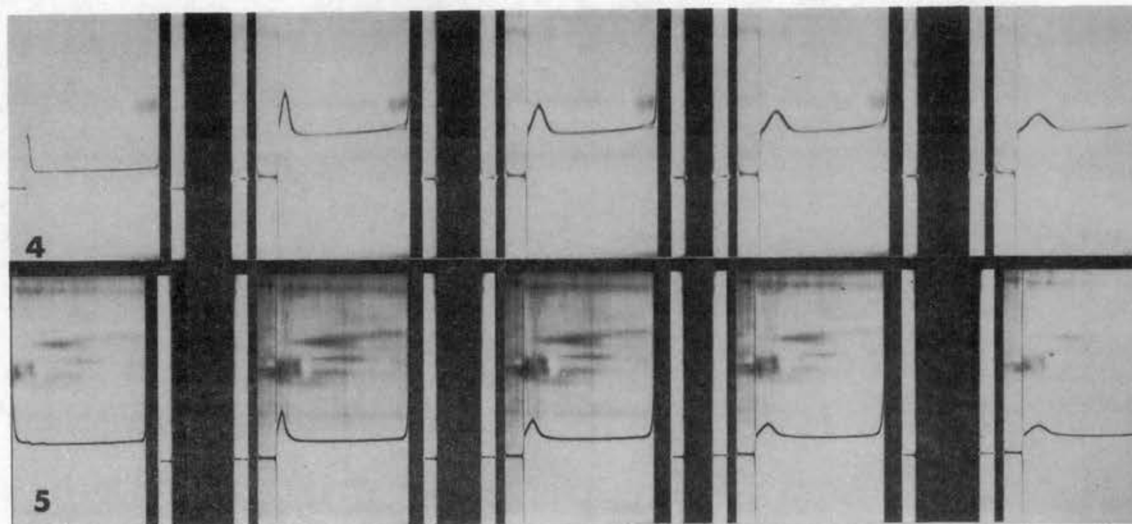


Figure 4. Sedimentation patterns of membrane subunits prepared by sodium lauryl sulfate.

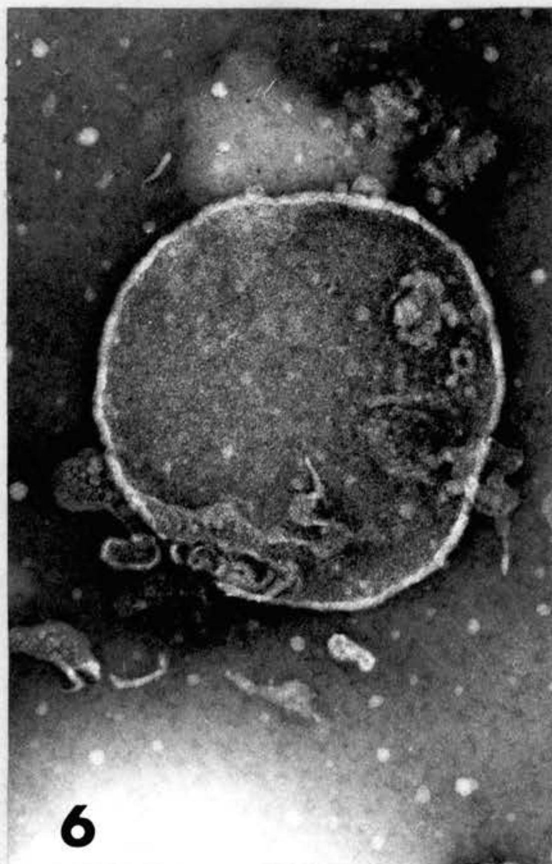
Figure 5. Sedimentation patterns of membrane subunits prepared by sonic disruption.

Figure 6. Whole Membrane Negatively Stained with Uranyl Acetate (68,000X)

Figure 7. Reaggregated Membrane Negatively Stained with Phosphotungstic Acid (68,000X)

Figure 8. Thin Sections of Whole Membranes (130,000X)

Figure 9. Thin Sections of Reaggregated Membranes (68,000X)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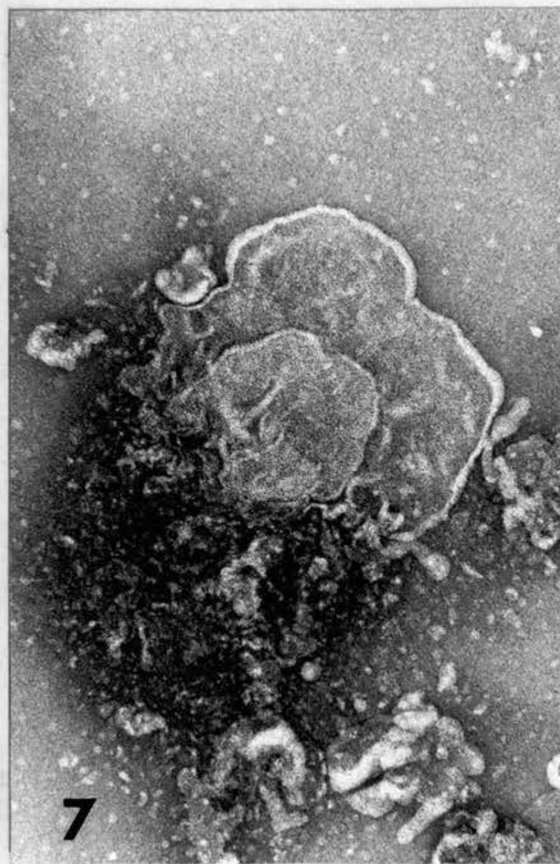
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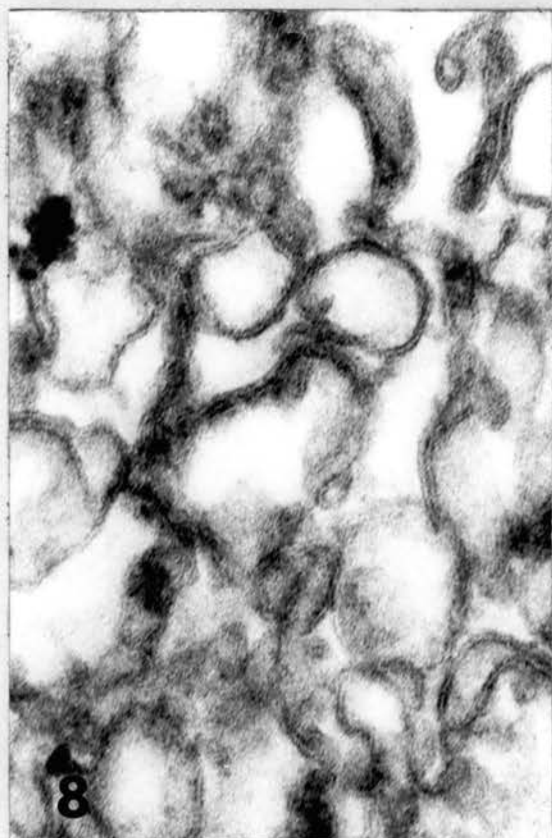
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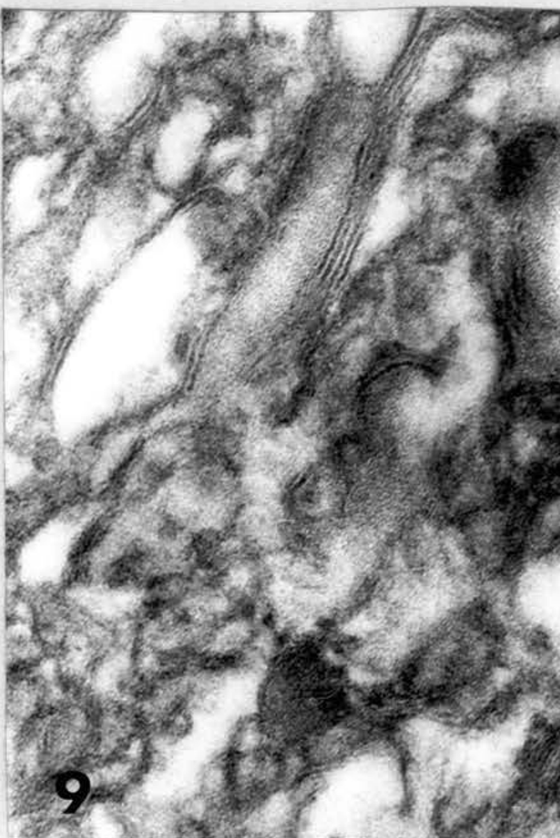
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Thin-sectioning of reaggregated membrane material reveals that the subunits are reconstituted into a three-layered structure much like the original membrane (Figure 9).

A suspension of whole membranes was subjected to sonic disruption (Branson Sonifier) to determine if a subunit form could be obtained by this type treatment. Sonic disruption was performed at maximum power output for fifteen seconds followed by a two minute cooling period in an ice bath. After eight sonic bursts, the suspension was transparent and appeared much like that obtained using SLS. After centrifugation (30 minutes at 70,000 x G) no pellet was observed indicating disruption of the membranes to a subunit form. These subunits were dialyzed for twelve days during which time no visible signs of reaggregation were evident (fluff formation); however, after centrifugation (30 minutes at 70,000 x G) a small amount of pellet was obtained. Therefore, it appears that although sonic disruption can also reduce membranes to a subunit form, the time required for minimal reaggregation (twelve days) is excessive and indicates that this treatment damages the subunit in such a way that reaggregation is greatly impeded.

Ultracentrifugation (Model E) of subunits obtained by sonic disruption was performed and revealed the presence of a uniform sized particle with an average sedimentation constant = 2.1 Svedberg units (Figure 5). This subunit is definitely smaller than that obtained using sodium lauryl sulfate and probably indicates that some critical component(s) is stripped off during disruption, thus, precluding reaggregation under our conditions. It is also possible that sonic disruption causes sufficient protein denaturation to impede reaggregation.

Effect of Several Cations on Membrane Reaggregation

After it was established that magnesium was necessary for membrane subunit reaggregation, the next logical step was to determine if other metal ions would function similarly in the system. The divalent cations, manganese, calcium, and zinc, and the trivalent metal cations, iron and aluminum, were used to replace magnesium in the dialysis system at the same concentration (0.01 M). After four days of dialysis, optical density readings were obtained; data are recorded in Table VII.

Dialysis in the presence of calcium caused rapid precipitation of residual detergent in the dialysis bag. After a few hours, the bag was re-opened and the precipitate removed by centrifugation; the supernatant, containing membrane subunits, was then re-dialyzed.

Table VII indicates that all divalent cations tested will permit reaggregation of membrane subunits. However, all the metal cations but magnesium contaminated reaggregated membrane with a detergent-metal precipitate. Absorption spectra of the resuspended pellets confirmed that carotenoids entered all reaggregates formed in the presence of divalent metals.

Trivalent cations produced a waxy precipitate which could not be uniformly resuspended for optical density readings. Absorption spectra of the pellets obtained in the presence of trivalent cations revealed very little if any carotenoid material (Figure 10).

The Effect of Various Chemical Agents on Membrane Reaggregation

Various chemical agents, reported to have a primary or secondary site of action on the membrane (see introduction) were added to the

TABLE VII

EFFECT OF SEVERAL CATIONS ON MEMBRANE SUBUNIT REAGGREGATION

Cation	440 mu*	640 mu*	Appearance of Pellets
Mg (Control)	.37	.08	Yellow pellet
Mn	.90	.23	White precipitate with some yellow membrane.
Ca**	.48	.14	Yellow membrane with some white precipitate.
Mg (Control)	.32	.08	Yellow pellet
Zn	.98	.49	White precipitate with some yellow membrane.
Fe	Pellet would not resuspend for O.D. measurement		Waxy rust-colored precipitate.
Al	Pellet would not resuspend for O.D. measurement		Waxy white precipitate

* Optical density of reaggregated membrane pellet resuspended in 2.0 mls buffer.

** Sodium lauryl sulfate precipitated by calcium was removed by centrifugation.

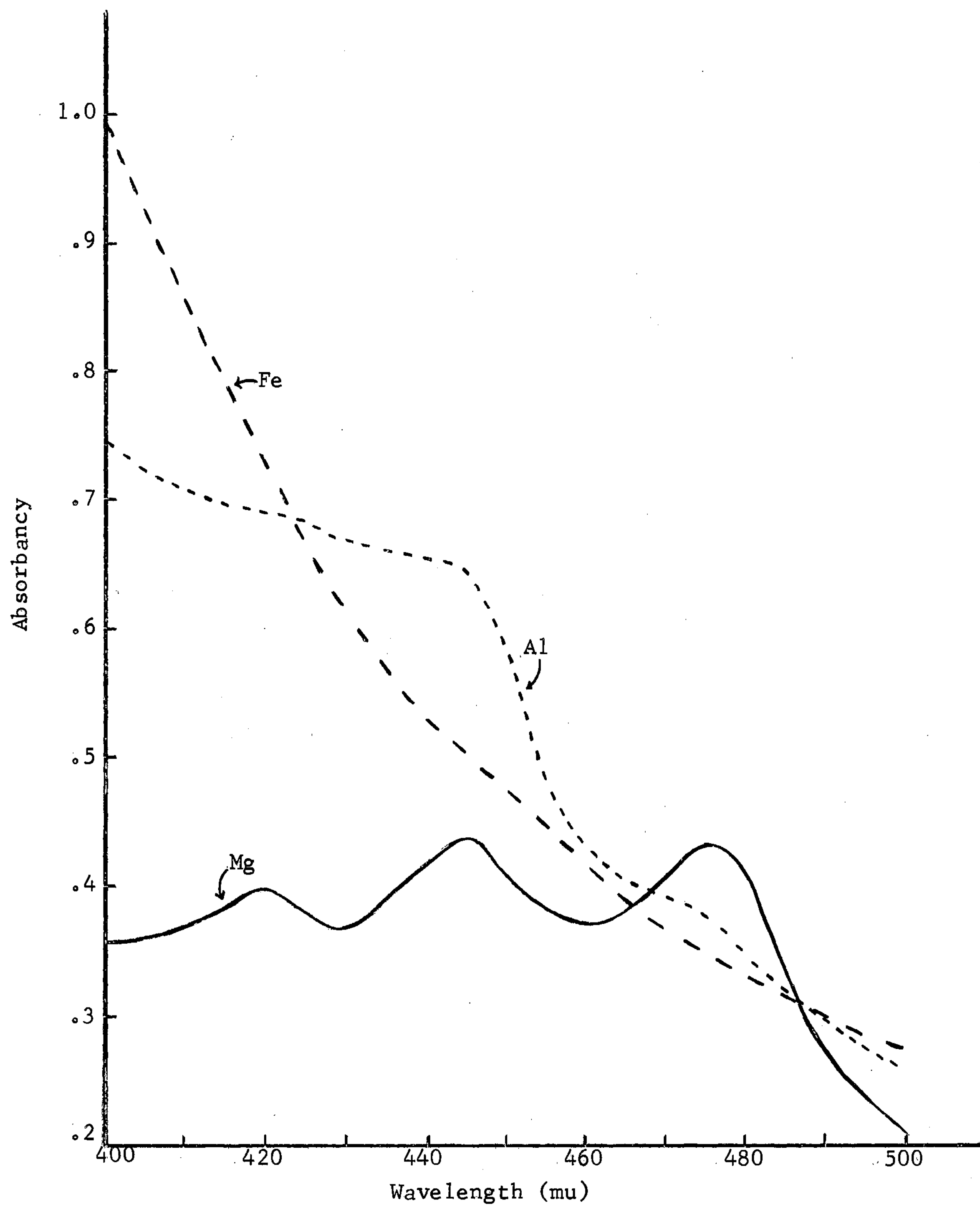


Figure 10. Absorption Spectra of Reaggregates Produced in the Presence of Trivalent Metal Ions

dialyzing solution to determine if an effect could be detected at the reaggregation level. Duplicate situations without magnesium were set up with each compound to determine if reaggregation might be induced by the compound(s) under test in the absence of the metal ion. These results are presented in Table VIII.

Penicillin at 100, 1000, and 5000 units per ml was added to the dialyzing system with no apparent alteration (inhibition) in the reaggregating process. However, at all concentrations of the antibiotic, an increased yield of reaggregated membrane material was noted when compared to control situations (Table VIII). Also, reaggregation occurred more quickly than in controls. Penicillin could not replace magnesium in the reaggregation system.

Vancomycin and pantoyl lactone, like penicillin, also showed no noticeable inhibition of membrane reaggregation. No significant differences in absorption were noted when membrane material formed in their presence was compared to controls. These compounds also lacked the ability to cause or allow reaggregation of membrane subunits when magnesium was omitted.

Diphenylamine, which alters carotenoid synthesis, was solubilized in ethanol and added to the dialyzing system. An additional control containing the same amount of ethanol was included in the experiment. Membrane subunits reaggregated in the presence of diphenylamine, formed a loosely packed pellet after centrifugation; however, the pellet material contained carotenoid. A slight increase in optical density was observed and was probably due to detergent precipitation. Diphenylamine could not induce reaggregation without magnesium (Figure 11). Ethanol, at the one concentration tested, did not precipitate sodium lauryl

TABLE VIII

THE EFFECT OF SEVERAL CHEMICAL AGENTS ON MEMBRANE SUBUNIT REAGGREGATION

Compound	O.D. Supernatant 440	O.D. Supernatant 640	O.D. Resuspended Pellet 440	O.D. Resuspended Pellet 640
Penicillin				
100 u/ml+Mg	.04	0	.25	.16
100 u/ml-Mg	.07	0	no pellet	
1000 u/ml+Mg	.04	0	.25	.18
1000 u/ml-Mg	.08	0	no pellet	
Control+Mg	.04	0	.21	.11
Control-Mg	.05	0	no pellet	
5000 u/ml+Mg	.04	0	.65	.25
5000 u/ml-Mg	.07	0	.05	.02
Control+Mg	.05	0	.45	.19
Control-Mg	.08	0	.02	.01
Vancomycin				
175 ug/ml+Mg	.04	0	.57	.23
175 ug/ml-Mg	.13	0	no pellet	
Control+Mg	.09	0	.61	.19
Control-Mg	.15	0	no pellet	
Pantoyl Lactone				
0.01 M+Mg	.03	0	.27	.12
0.01 M-Mg	.04	0	no pellet	
Control+Mg	.05	0	.21	.11
Diphenylamine				
25 ug/ml+Mg	.05	0	.68	.27
25 ug/ml-Mg	.17	0	no pellet	
Ethanol+Mg	.06	0	.54	.18
Ethanol-Mg	.15	0	no pellet	
Control+Mg	.09	0	.61	.19
Control-Mg	.09	0	no pellet	

TABLE VIII (Continued)

Compound	O.D. Supernatant 440	O.D. Supernatant 640	O.D. Resuspended Pellet 440		O.D. Resuspended Pellet 640
Putrescine					
0.01 M+Mg	.05	0	.62		.51
0.01 M-Mg	.09	0	.36	ppt.	.20
Control+Mg	.04	0	.52		.32
Control-Mg	.08	0	.02		.04
Spermine					
0.01 M+Mg	Could not be resuspended for O.D. readings				
0.01 M-Mg	Could not be resuspended for O.D. readings				
Control+Mg	.04	0	.52		.32
Control-Mg	.08	0	.02		.04

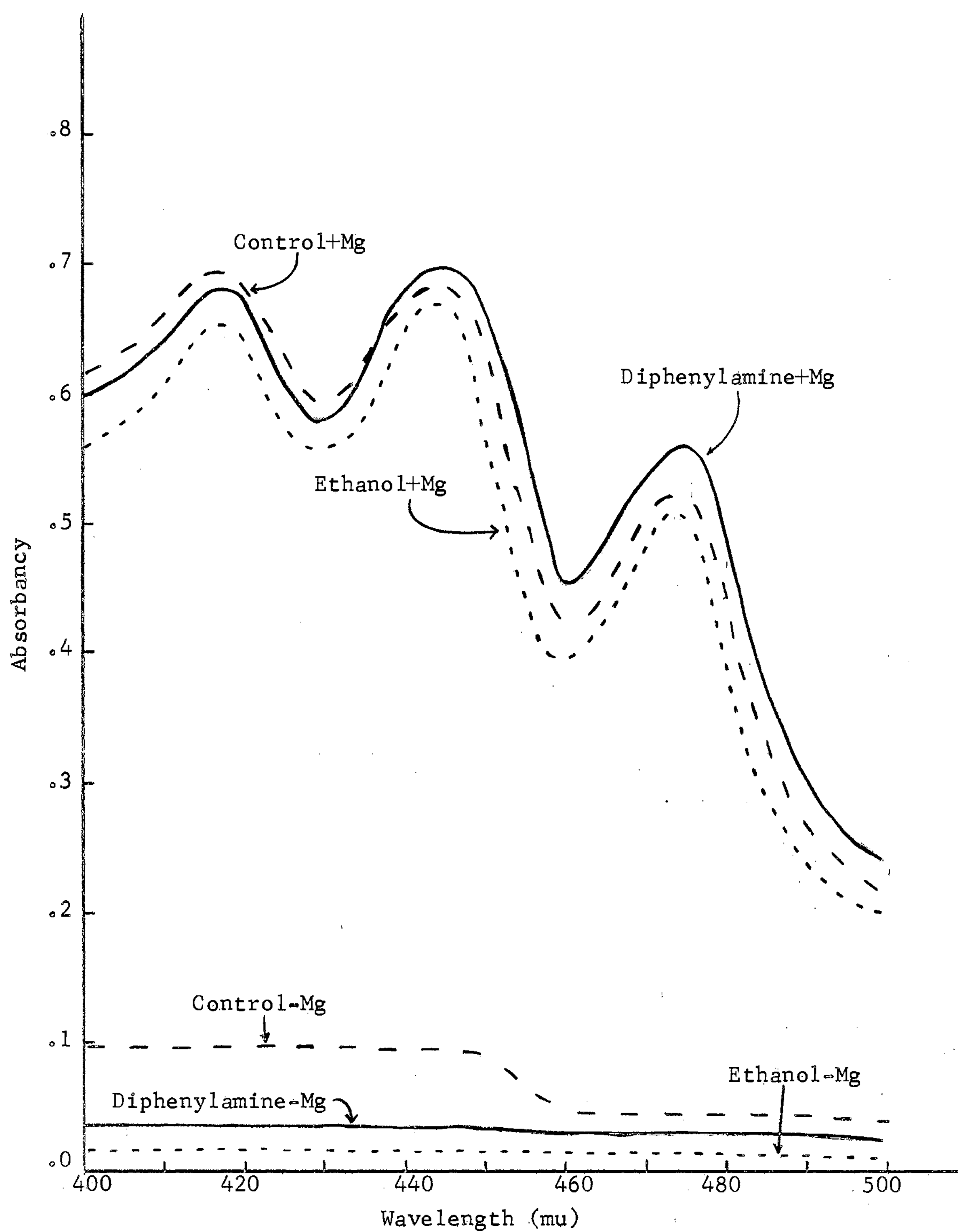


Figure 11. Absorption Spectra of Membranes Reaggregated in the Presence of Diphenylamine and Ethanol

sulfate nor did it have an observable effect on reaggregation. Absorption spectra of reaggregates formed in the presence of diphenylamine and ethanol (Figure 11) did not differ from those normally obtained.

Razin, Morowitz, and Terry (1965) reported that spermine could replace magnesium in their reaggregating system utilizing membrane subunits prepared from Myco. laidlawii. When putrescine and spermine were added to the M. lysodeikticus reaggregating system, a precipitate with sodium lauryl sulfate formed in the dialysis bag. This precipitate was sedimented by low speed centrifugation prior to high speed centrifugation which was then used to obtain the membrane material formed in the presence of spermine or putrescine. Membranous material formed in the presence of spermine did contain carotenoids, but this material was aberrant since it could not be resuspended to obtain optical density readings. The carotenoid-containing pellet obtained in the presence of putrescine was resuspended without difficulty. In the absence of magnesium spermine, but not putrescine, was able to initiate the aberrant reaggregation reported above. Although spermine can replace Mg^{++} ions to cause reaggregation of our subunits, the membranous material formed is too tightly bonded and appears to exist as a large precipitate that cannot be broken into individual membrane sheets. This particular effect is similar to that obtained using trivalent ions.

Effect of Growth Environment on Membrane Reaggregation

Because compounds such as penicillin, diphenylamine, etc. did not inhibit reaggregation of membrane subunits, it was decided to grow the organism in the presence of inhibiting concentrations of some of these compounds, isolate the membranes, disaggregate them into subunits, and,

finally, test the ability of these subunits to reaggregate in our normal system.

Diphenylamine, which alters synthesis of carotenoid pigment, was added to 100 ml medium in one ml ethanol (final concentration 175 ug/ml). The same amount of ethanol without diphenylamine was added to another flask as a control. Diphenylamine, in addition to causing production of nonpigmented cells, also inhibits growth of the organism (Table VIII). Membranes from cells grown in the presence of diphenylamine appeared to disaggregate in the normal way and a white fluff was produced after dialysis for six days. A pellet having a waxy consistency was obtained after centrifugation and differed from control membranes only in absence of pigment. Although absorption readings at 440 mu were low (non-pigmented reaggregated membrane material), readings at 640 mu revealed reaggregation nearly equal to the control. Therefore, it was concluded that any membrane damage occurring as a result of growth in the presence of diphenylamine was not sufficient to cause inhibition in reaggregation. Ethanol inhibited growth to a small extent but membrane subunits prepared from these cells also reaggregated in the normal manner.

M. lysodeikticus was also grown in the presence of 0.07 ug/ml mitomycin c and membrane subunits prepared from these cells. Although inhibition of growth was noted (Table IX), reaggregation of membrane subunits made from these antibiotic-treated cells was not inhibited.

D-Serine (5×10^{-4} M) was another compound added to the defined medium of M. lysodeikticus and membrane subunits prepared in the normal manner. Growth inhibition at this level of D-serine is high and required 1600 ml of cells to prepare sufficient membranes for testing

TABLE IX

EFFECT OF GROWTH ENVIRONMENT ON MEMBRANE SUBUNIT REAGGREGATION

Situation	Whole Cells*	Membrane Subunit		Resuspended Pellet	
	540 mu	440 mu	640 mu	440 mu	640 mu
Diphenylamine	0.10	0.07	0.01	0.43	0.26
Ethanol	0.23	0.24	0.01	0.50	0.27
Control	0.27	0.30	0.01	0.51	0.29
Mitomycin	0.27	0.20	0.01	0.51	0.29
Control	0.31	0.26	0.01	0.56	0.34
D-Serine	0.06	0.18	0.01	0.24	0.18
Control	0.41	0.29**	0.01	0.26	0.19

* Denotes growth of the organism.

** Control membrane subunits were diluted to the same concentration as the D-serine subunits (0.08 O.D.) so that equal amounts were dialyzed.

(Table IX). The control suspension of subunits was diluted to equal the concentration of D-serine subunits and dialysis allowed to proceed for five days. Nearly equal amounts of reaggregated membrane material were obtained from both situations indicating that growth in the presence of D-serine does not affect the membrane sufficiently to inhibit subunit formation and subsequent aggregation.

Bonding Involved in Membrane Reaggregation

During reaggregation the subunits must bond together with the aid of magnesium to form the membrane-like sheets. To obtain data relative to the type of bonding involved, several agents known to disrupt specific types of bonds were tested.

The role of hydrogen bonding in reaggregated membrane material was examined using urea and guanidine; agents known to disrupt hydrogen bonds. Urea (8.0 M in 1:20 Beta buffer) was added to reaggregated membranes at 25° C and at sixty minute intervals optical density readings were made at 640 mu (Table X). An immediate effect of urea was noted because a significant reduction in optical density occurred when compared to control. Also, visual observations revealed an orange membrane suspension after urea treatment rather than the customary yellow. It appears that urea affects primarily the optical properties of the suspension causing a reduction in density and change in color rather than membrane disaggregation, since centrifugation of the urea-treated suspensions yields an orange pellet with little or no change in total mass.

TABLE X

EFFECT OF UREA AND GUANIDINE ON REAGGREGATED MEMBRANE STRUCTURE

	O.D. 640 mu			After
	0	1 Hr.	2 Hr.	Centrifugation
Control	.28	.27	.26	.24
Urea (8.0 M)	.15	.13	.13	.12
Control	.26	.25	.25	.22
Guanidine (5.0 M)	.34	.33	.33	.31

Guanidine-HCl (5.0 M in 1:20 Beta buffer) was found to have no effect either on optical density or centrifugation properties of reaggregated membrane.

From these studies, it was concluded that hydrogen bonding is not the primary or most important binding mode involved in holding the reaggregated membrane sheets together.

A possibility existed that magnesium was responsible for holding membrane subunits together and removal of the metal would cause loss of membrane structure. Therefore, a chelating agent, ethylenediaminetetracetic acid (0.1 M in 1:20 Beta buffer), was added to reaggregated membrane at 25° C and optical density readings made every thirty minutes (Table XI). After an initial reduction at thirty minutes, both control and EDTA-treated reaggregated membranes showed similar optical density properties and equally large amounts of pellet material could be obtained from these treated reaggregated membranes. Therefore, it is most probable that if magnesium acts to bind subunits together, the magnesium is buried within the reaggregated membrane sheets and cannot

be removed by a chelating agent sufficiently to cause disaggregation.

TABLE XI

EFFECT OF EDTA ON REAGGREGATED MEMBRANE STRUCTURE

	O.D. 640 mu			After
	0 minutes	30	60	Centrifugation
Control	.28	.27	.26	.24
EDTA	.29	.25	.25	.24

Reaggregated membranes were also washed in distilled water by centrifugation to determine if removal of salts would cause disaggregation. A pellet of reaggregated membrane was obtained after each wash and, after resuspension in equal volumes of water, optical density remained equal to that obtained in buffer. Therefore, once the membrane subunits are in the reaggregated state, salts are not necessary to hold them together.

Razin, Morowitz, and Terry (1965) speculated that the bonding mode in reaggregated PPLO membranes was apolar or hydrophobic. This type of bonding requires exclusion of water molecules for close association of hydrophobic regions.

Because SLS caused disaggregation either of whole or reaggregated membranes, it appeared most likely that bonding in the membrane of M. lysodeikticus also involved hydrophobic groups (van der Waals forces). Since SLS is an anionic detergent, other detergents (cationic and non-ionic) were compared to determine disaggregating ability. Compounds tested included Tween 80 (polyoxyethylene sorbitan monoleate)

a non-ionic detergent, and two cationic detergents G3436A (unknown chemical composition) and G-271 (N-soya n-ethyl morpholinium ethosulfate) provided by Atlas Chemcial Company. Final concentration of all detergents in the reaction mixture was 8.65 mg/ml based on a 0.3 M solution of sodium lauryl sulfate. Of all the compounds tested, only SLS reduced the membrane suspension to the subunit form. The non-ionic detergent Tween 80 had no effect, and both cationic detergents caused formation of a heavy white precipitate without membrane disruption.

It was possible that a high hydrogen ion concentration (the tests reported above were performed at pH 7.4 in Beta buffer) would prevent precipitation of the cationic detergent and allow membrane disruption. To test this possibility both whole and reaggregated membranes were suspended in a sodium acetate-acetic acid buffer at a pH of 4.0 in the presence of G3436A and G-271 cationic detergents. The low pH did prevent detergent precipitation and surprisingly it was observed that reaggregated membranes are almost completely disaggregated by the G3436A detergent. Although there is some effect on the whole membrane, it is not as extensive as that which occurs using reaggregated membrane sheets (Table XII).

These are the only data obtained which indicate that a difference exists between structuring of reaggregated membrane material and whole membranes.

In order to determine if any further differences could be demonstrated between whole membranes and reaggregated membrane material, each was subjected to trypsin digestion (10 ug/ml) in ammonium bicarbonate buffer (3.95 mg/ml) at 37° C without prior denaturation of protein material (not heated). After three hours, no change in optical

TABLE XII

EFFECT OF CATIONIC DETERGENTS ON WHOLE AND REAGGREGATED MEMBRANES AT pH 4.0

Situation	O.D. at 640 mu*	Percent Change
Whole Membrane		
Control	.42	0%
G271	.46	+ 9%
G3436A	.33	-21%
Reaggregated Membrane		
Control	.25	0%
G271	.40	+60%
G3436A	.14	-44%

* Reading after 30 minutes reaction time.

density was observed in either preparation.

It appeared possible that during subunit formation, detergent caused removal of phospholipids and carotenoids. If this happened, it would be necessary for these compounds to be reconstructed with protein prior to or during aggregation into membrane sheets because evidence has been presented showing that reaggregated membrane material contains proteins, phospholipids, and carotenoids. If the phospholipids or carotenoids were freed from protein by detergent action, they should migrate from water where they are insoluble into chloroform where they are soluble. This possibility was tested by layering detergent-produced subunits over chloroform for 16 hours under an atmosphere of nitrogen. In no case did carotenoids or phospholipids migrate into the chloroform phase. It was also noted that subunits exposed to the chloroform treatment reaggregated normally when compared to subunits not exposed to chloroform.

A further type of experiment relative to this point was also done. Membrane subunits prepared using SLS were spotted on a thin-layer plate (silica gel) and developed (ascending) in chloroform. In no instance could we detect movement from the origin either of carotenoid pigments or phospholipids.

These experiments suggest that the membrane subunit freed by detergent action exists as a phospholipid-protein-carotenoid complex.

Two further aspects were studied relating to the entire problem. First, we wished to obtain evidence concerning the molecular architecture of the membrane. According to the Danielli-Davson unit-membrane hypothesis (1935), the membrane exists as a three-layered unit structure (phospholipid sanwiched between two layers of protein). If this is

true, then removal of phospholipids should cause collapse of the structure and this alteration should be easily visualized in cross-sections using the electron microscope.

To accomplish removal of phospholipids and carotenoids, four solvents (ethanol, methanol, ethyl acetate, and acetone) were selected. Each was reacted with whole membrane at 4° C (system pre-cooled) for 30 minutes. After extraction, the material remaining was collected by centrifugation and all supernatants were concentrated under nitrogen. The extracted membranes were then viewed using the electron microscope for gross anatomy and in thin-section. Pictorial data relating gross anatomy are presented in Figures 12 through 19.

As seen in these pictures all of the extraction procedures caused distortion of the cell membrane. It could be concluded that the clear areas in the membrane represent areas where phospholipid has been removed. This conclusion is not tenable, however, because ethyl acetate does not remove any phospholipids or carotenoid yet this solvent causes the same type of anatomical distortion as treatment with methanol, ethanol, and acetone which do remove phospholipids and carotenoids (Figure 20). Because of the fortunate inclusion of ethyl acetate in this experiment, we conclude that the membrane distortion shown in Figure 14 is due to precipitation and shrinking of membrane components (primarily protein).

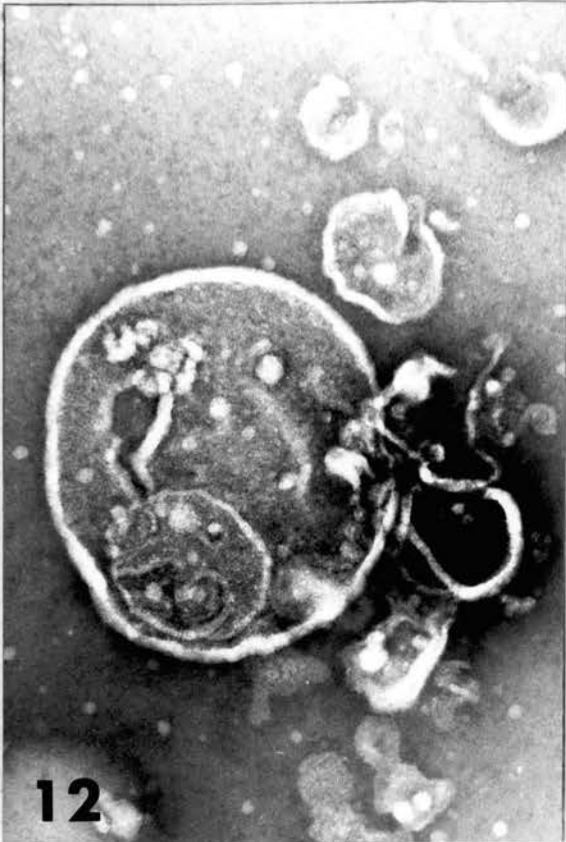
Pictorial data obtained from viewing thin-sections reveals that even though phospholipids and carotenoids are removed the sandwich structure (three layered) of the membrane is retained (Figures 17 through 19). Because of these data, it would appear that Green and Hechter (1965) are justified in their criticism of the unit membrane

Figure 12. Whole Membrane Control Negatively Stained with Uranyl Acetate (68,000X)

Figure 13. Methanol Extracted Membranes Negatively Stained with Uranyl Acetate (68,000X)

Figure 14. Ethyl Acetate Extracted Membranes Negatively Stained with Uranyl Acetate (68,000X)

Figure 15. Acetone Extracted Membranes Negatively Stained with Uranyl Acetate (68,000X)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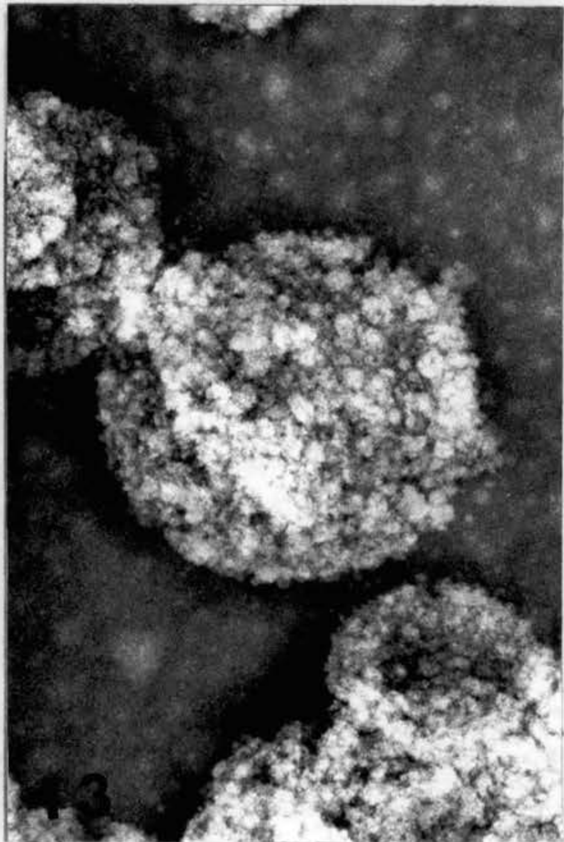

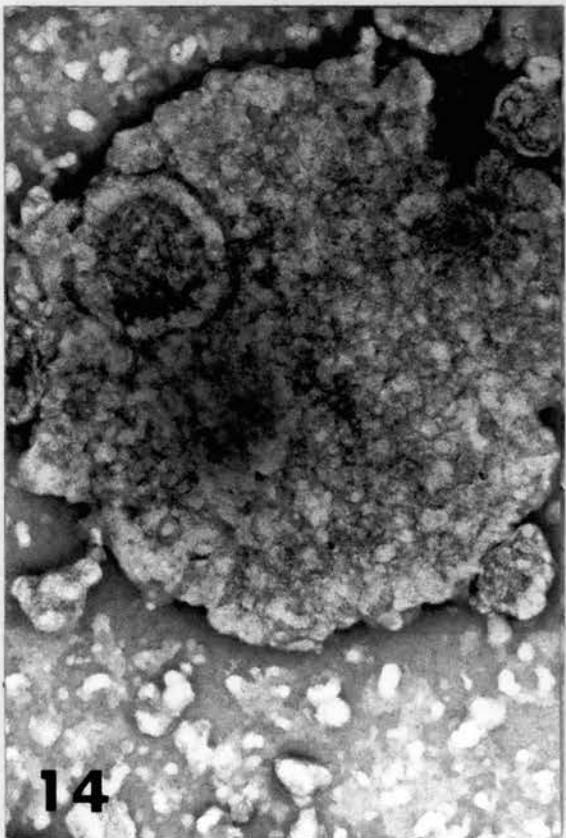
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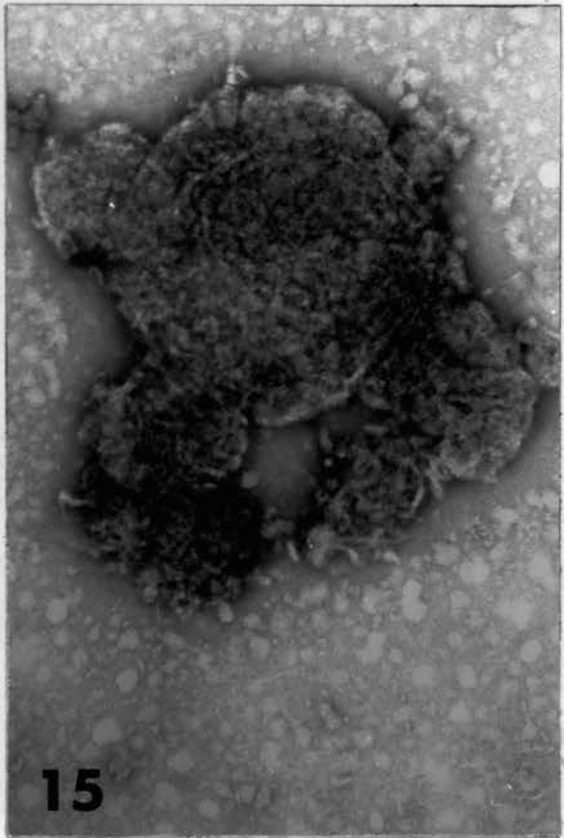
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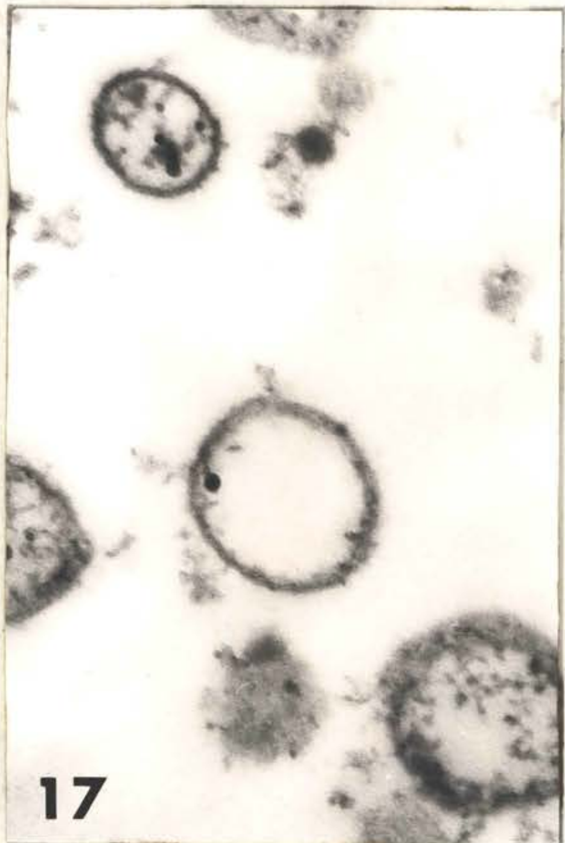
Figure 16. Thin Section of Whole Membranes (130,000X)

Figure 17. Thin Section of Methanol Extracted Membranes (68,000X)

Figure 18. Thin Section of Ethyl Acetate Extracted Membranes (68,000X)

Figure 19. Thin Section of Acetone Extracted Membranes (68,000X)

16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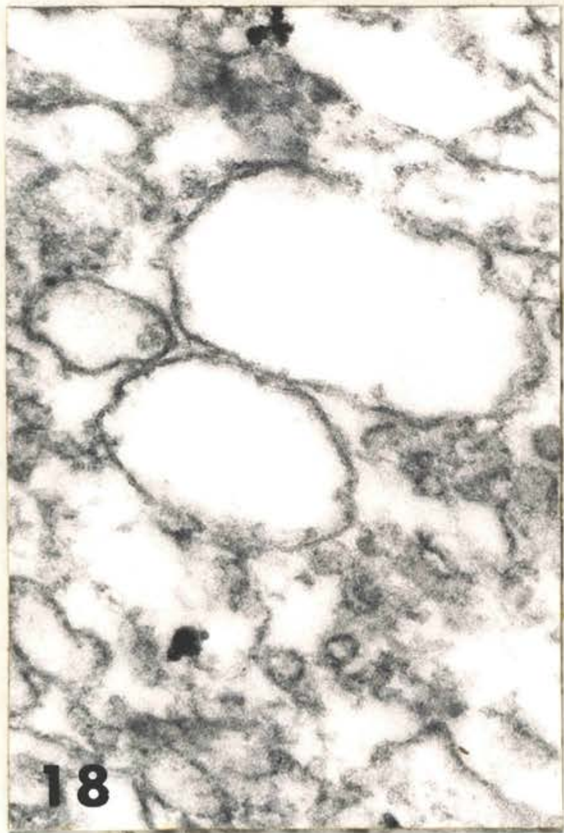
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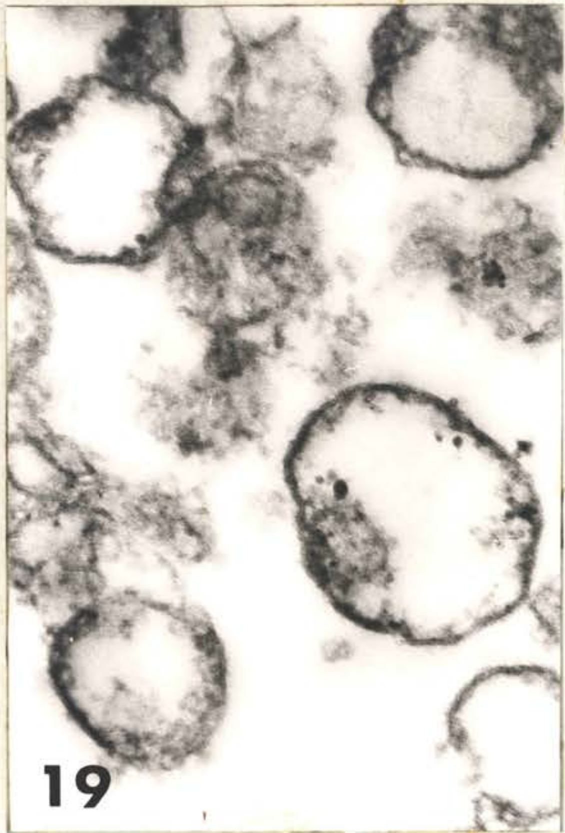
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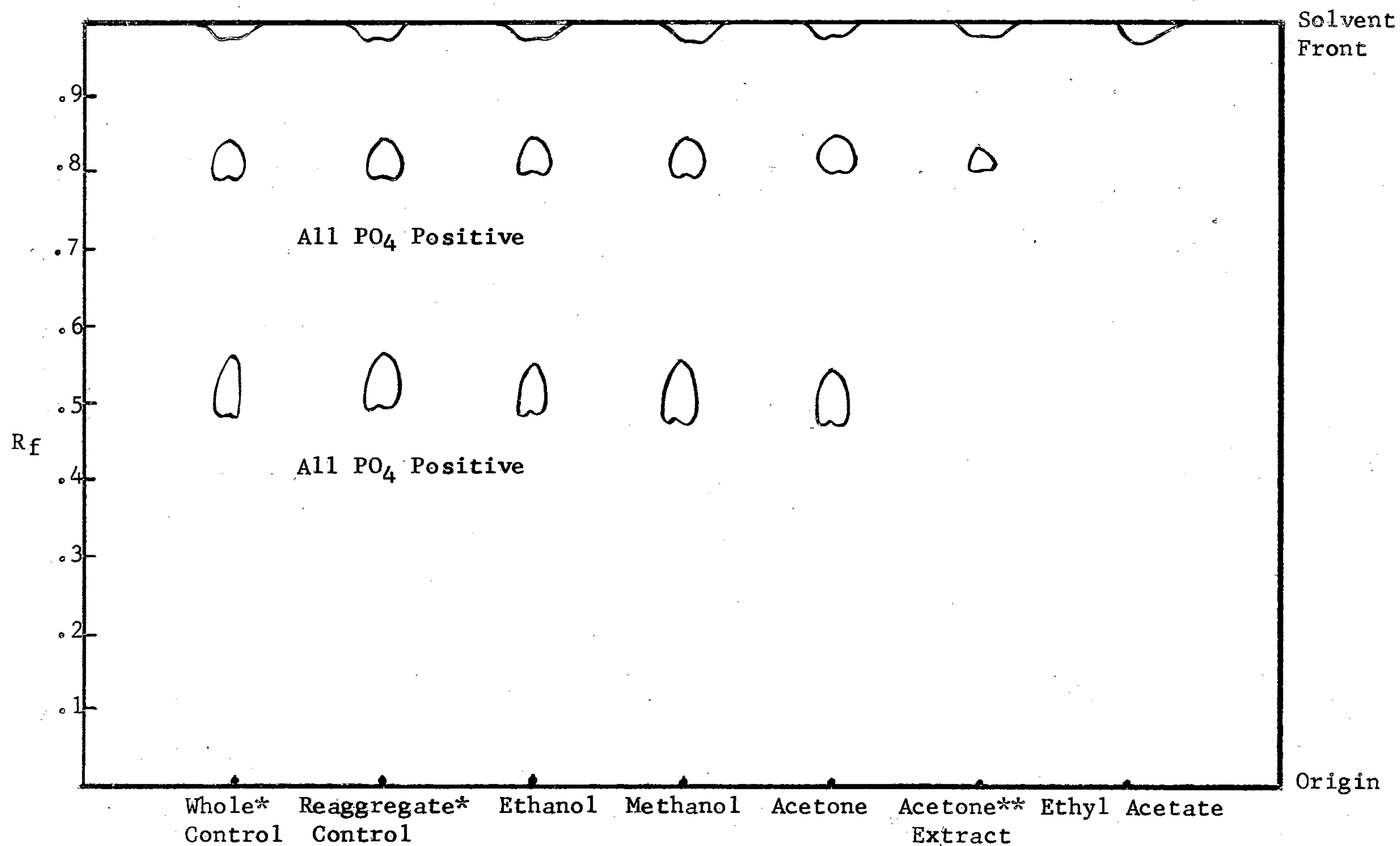


*Control = Extracted with chloroform:methanol (2:1)
**Chloroform:methanol extraction of acetone precipitated membranes
Solvent system: Chloroform-methanol-water (65:25:4)

Figure 20. Phospholipid Patterns of Various Membrane Extracts

model structure proposed by Danielli and Davson.

The second area in which we wanted to obtain data related to the need for phospholipid during disaggregation and reaggregation. Using the same approach as given above, it could be demonstrated that the precipitated residual of membrane material obtained after solvent extraction (primarily protein) is bonded through hydrophobic groups because SLS causes an immediate and complete disaggregation of this material. This finding is very important and indicates that detergent action can dissociate membrane "protein to protein" units of some type (into monomeric units?). Therefore, phospholipids and carotenoids are not necessary nor are they the focal point for attack by detergent during membrane dissolution. This information coupled with data obtained from the chloroform solubility and ultracentrifugation experiments allows us to present the hypothesis that phospholipids and carotenoids are probably associated with protein through a weak type of bonding (van der Waals forces). This type bonding can be broken by relatively polar and water miscible solvents such as acetone and alcohols (methanol or ethanol) because these solvents can enter the highly charged area, solubilize the lipids, and cause precipitation of the protein membrane continuum. The protein molecules forming the membrane continuum are also bonded to each other via hydrophobic groups (van der Waals forces), but this charged area can be entered, wetted, and disaggregated into a subunit form only by highly charged bifunctional molecules containing large water shells. Therefore, the basal continuum of the cell membrane of M. lysodeikticus is a highly charged protein to protein structure held together primarily through van der Waals forces (hydrophobic groups) having phospholipids and carotenoids

in very close physical association as a type of added superstructure.

If the above hypothesis is correct, then phospholipids and carotenoids should not be essential for reaggregation. To study this aspect of the problem, phospholipid-carotenoid denuded membranes (solvent extracted) were dissociated with SLS and tested for reaggregating ability in the presence and absence of phospholipids and carotenoids. (phospholipids and carotenoids were tested by adding back to the dialysis bag the extracted and concentrated phospholipids and carotenoids).

In the absence of added phospholipids and carotenoids, subunits obtained after ethyl acetate or ethanol extraction reaggregate poorly. Anatomically they do not have the appearance of membrane sheets, but, instead, appear as individual clumps of relatively amorphous material which exhibit a tendency to aggregate or stick to each other (Figures 23 and 24).

Reaggregation after extraction with ethyl acetate is extremely poor even though, as shown in Figure 20, phospholipids and carotenoids are not removed by ethyl acetate. Although reaggregation after prior alcohol extraction is poor, the material that reaggregates possesses the typical three-layered (unit membrane) structure when viewed in cross-section (Figures 25, 26, and 27).

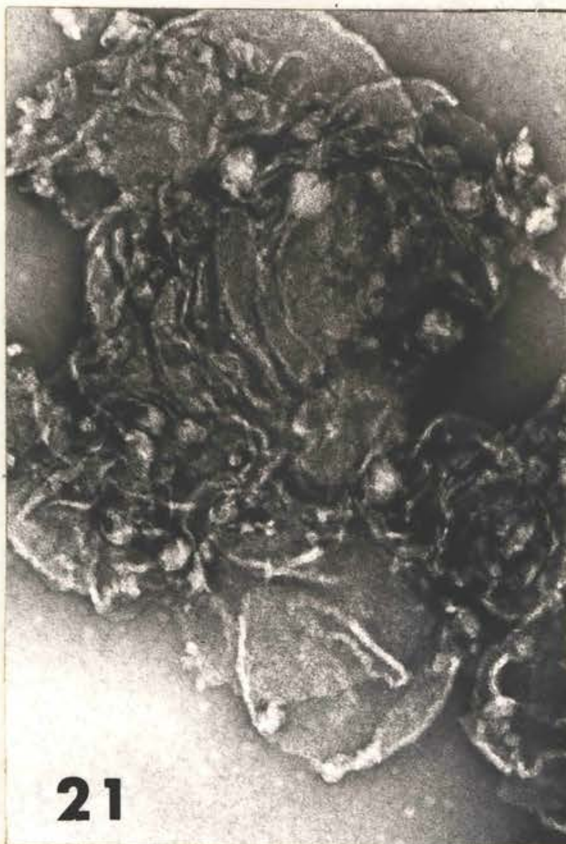
Data obtained using acetone extraction are extremely interesting. Reaggregated sheets are constructed from the residual protein and these sheets appear to be more like normal sheets than any others obtained after extraction (Figure 21). They also exhibit the typical three-layered unit membrane structure when viewed in cross-section (Figures 25 and 26). Further, after centrifugation, the pellet obtained

Figure 21. Reaggregates of Acetone Extracted Membranes: Negatively Stained with Phosphotungstic Acid (68,000X)

Figure 22. Reaggregates of Acetone Extracted Membranes with Phospholipid Added Back: Negatively Stained with Phosphotungstic Acid (68,000X)

Figure 23. Reaggregates of Ethanol Extracted Membranes: Negatively Stained with Phosphotungstic Acid (68,000X)

Figure 24. Reaggregates of Ethyl Acetate Extracted Membranes: Negatively Stained with Phosphotungstic Acid (68,000X)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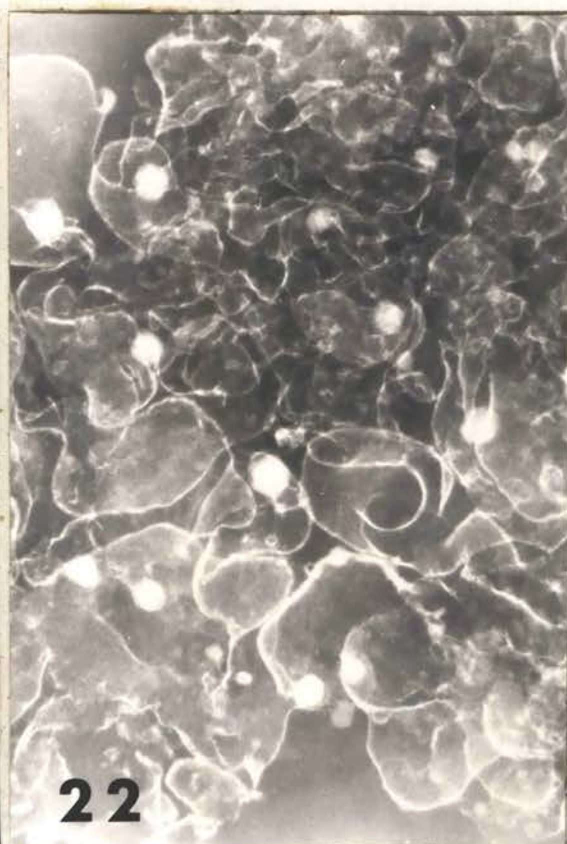
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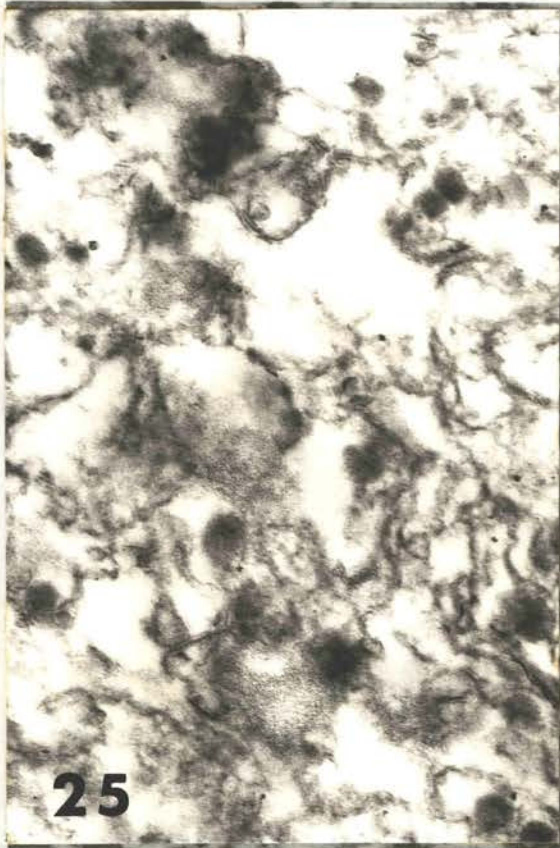
Figures 25 and 26. Thin Sections of Reaggregates from Acetone Extracted Membranes (68,000X)

Figure 27. Thin Sections of Reaggregates from Acetone Extracted Membranes with Phospholipids Added Back (68,000X)

Figure 28. Thin Section of Reaggregates from Methanol Extracted Membranes (68,000X)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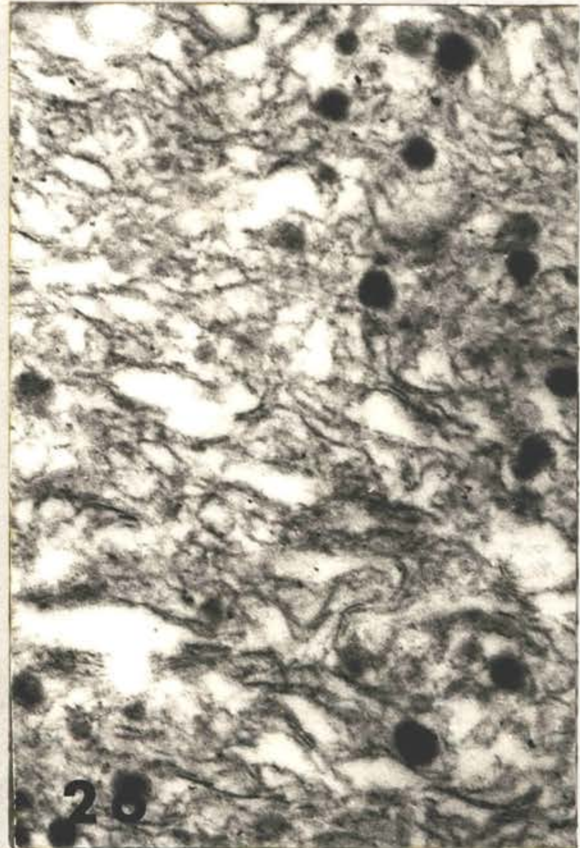

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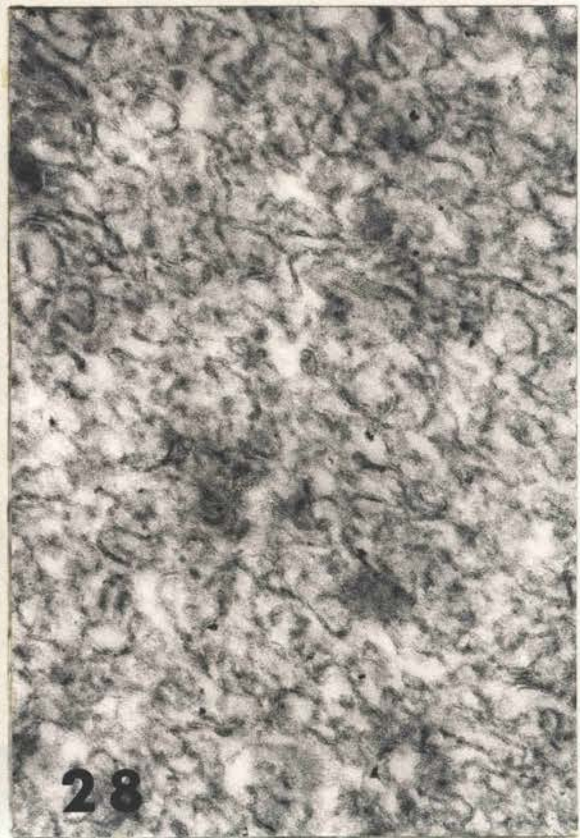
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presents a normal appearance because it is relatively transparent, shiny, and easily dispersed by pipetting. These membrane sheets are non-pigmented since carotenoids have been removed by the acetone treatment.

Reaggregation of subunits prepared after acetone extraction is good, but the amount of reaggregation is significantly increased if extracted phospholipids and carotenoids are added back to the system. Data relative to this point are given in Table XIII. It was evident from these experiments that membrane sheets obtained after adding back the extracted phospholipids and carotenoids are more "normal" appearing only in that they have reincorporated the carotenoid pigment, thus, yielding a yellow pellet after sedimentation (Figures 22 and 27).

This type of experiment also shows that phospholipids and carotenoids, even after being freed from protein subunits, can, under our conditions of dialysis, be reincorporated into the basal membrane structure. Were this not true, we could unequivocally state that disaggregation by SLS yields only a protein-phospholipid-carotenoid subunit.

Regardless of the experimentally observed result that addition of acetone-extracted phospholipids and carotenoids allows for construction of a pigmented and, therefore, more "normal" appearing membrane sheet, protein is the key compound that allows for disaggregation by detergent into a subunit form and these protein subunits, with or without phospholipids, and carotenoids, contain the needed information to allow reaggregation into a membrane sheet which can be viewed as the basal membrane continuum. The increased amount of reaggregation that occurs in the presence of added phsopholipids and carotenoids can probably be attributed to stabilization of the protein subunits by these compounds.

TABLE XIII

EFFECT OF PHOSPHOLIPID REMOVAL ON MEMBRANE REAGGREGATION

Extracted Membranes	O.D. at 640 mu Resuspended Reaggregate
Acetone Reaggregate	.13
Acetone Reaggregate + Phospholipid*	.49
Methanol Reaggregate	.04
Methanol Reaggregate + Phospholipid*	.28

* Extracted phospholipids and carotenoids concentrated and added back to the dialyzing bag.

A further point that can be made at this time is that protein subunits prepared using SLS after prior extraction with acetone do not reaggregate unless Mg^{++} ion is present in the dialyzing system. Therefore, this ion is not required for neutralization of the negative charges present on protein-associated phospholipids. Magnesium ions are probably required to neutralize and thus reduce negative charges present in protein molecules (reduce repulsion within the molecules and between molecules) thus maintaining their structure in the organized state necessary for recognition and aggregation.

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