"The author wishes to dedicate this thesis to his parents for their support and encouragement though-out the course of the study."

# A BIOCHEMICAL CHARACTERIZATION OF THE PLASMA MEMBRANE OF THE MYXAMOEBA AND SWARM CELLS OF THE MYXOMYCETE *DIDYMIUM IRIDIS*

by

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# ABSTRACT

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The plasma membrane of the myxamoeba and swarm cells of the Myxomycete *Didymium iridis* was biochemically characterized. Analysis of membrane proteins by SDS-polyacrylamide gel electrophoresis stained with Coomassie Blue revealed twelve components ranging in molecular weight from 24,000 - 200,000. The two slowest migrating components were also stained by the periodic acid - Schiffs (PAS) procedure indicating the presence of glycoproteins (GP) in these bands.

The lipid component was separated into major classes by column chromatography and further fractionated by thin-layer chromatography. Five phospholipids were resolved. Although two remain unidentified, it appears that phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), and phosphatidyl inositol (PI) are present while the lone glycolipid resolved is a derivative of monogalactosyldiacylglycerol. The membrane neutral lipid is present as sterol esters as shown by gas-liquid chromatography and infrared spectroscopy. The sterols most likely to be present include

## ACKNOWLEDGEMENTS

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# INTRODUCTION

In recent years the Myxomycetes have become increasingly popular organisms for use in biochemical research. They are easily maintained in cell culture, and have a fascinating, but problematic life cycle, both of which provide many areas of investigation. The life cycle of the Myxomycetes or the true, acellular slime molds, has been extensively detailed by Alexopoulos (1962), and by Gray and Alexopoulos in review (1968). (Plate 1).

Generally, the life cycle consists of a haploid or gametic stage, an assimilative plasmodial stage, and a plant-like fruiting stage. The gametic stage is characterized by the independent movement and multiplication of free-living myxamoeba which feed by the ingestion and digestion of bacterial cells. After an indefinite period of feeding and multiplication, the amoeba become transformed into gametes which fuse in pairs to form zygotes (Ross, 1967). The zygotes then develop by coordinated inflowing of the myxamoeba into multinucleate diploid plasmodia (Yemma and Therrien, 1972). Coalescence with other plasmodia can also occur thus contributing to an increase in size of the plasmodium. In this stage the organism is primarily assimilative and becomes increasingly plant-like in character, and after a period of migration, sporogenesis and spore formation takes place. Differentiation of the plasmodia

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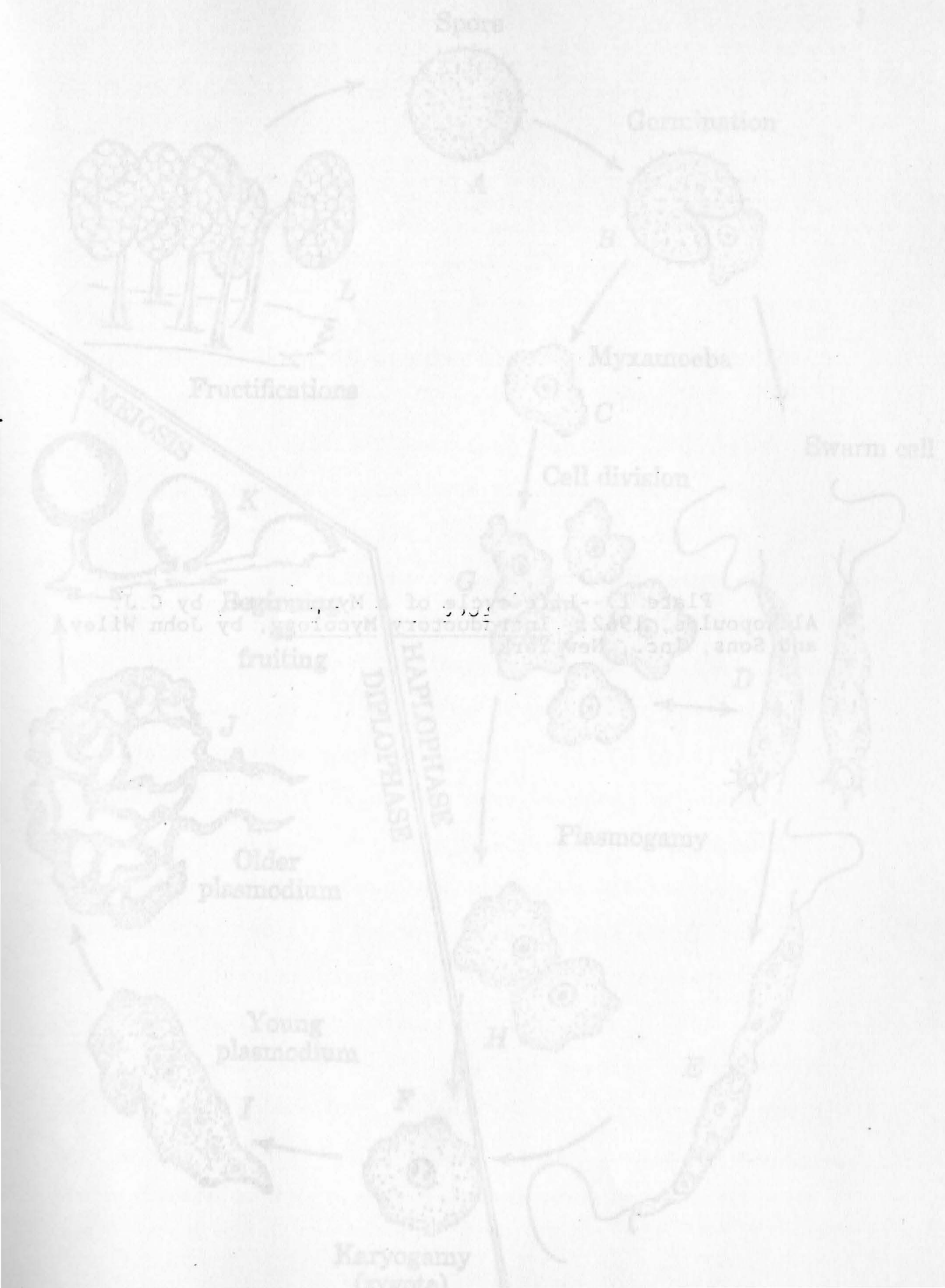
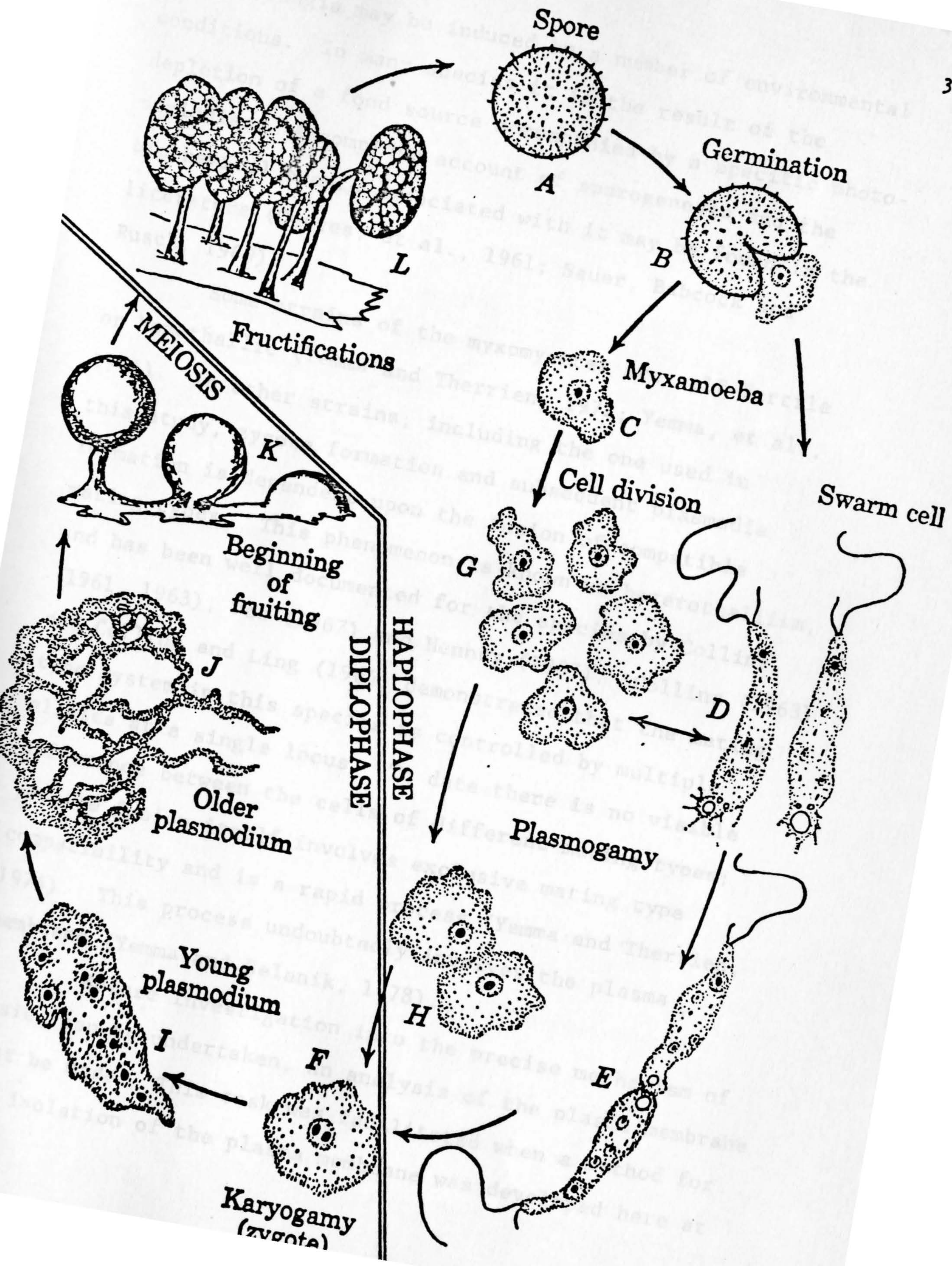



Plate I) -Life cycle of a Myxomycete, by C.J. Alexopoulos, 1962, Introductory Mycology, by John Wiley and Sons, Inc., New York


Spore
A
Germination
B
Myxamoeba
C
Cell division
G
Swarm cell
D
Plasmogamy
H
E
F
Karyogamy
(zygote)
Young
plasmodium
I
Older
plasmodium
J
Beginning
of
fruiting
K
MEIOSIS
Fructifications
L
DIPLOPHASE
HAPLOPHASE

into sporangia may be induced by a number of environmental conditions. In many species it is the result of the depletion of a food source accompanied by a specific photoperiodism. A complete account of sporogenesis and the biochemical events associated with it may be found in the literature (Guttes, et al., 1961; Sauer, Babcock and Rusch, 1969).

Some strains of the myxomycetes are self-fertile or homothallic (Yemma and Therrien, 1972; Yemma, et al., 1974). In other strains, including the one used in this study, zygote formation and subsequent plasmodia formation is dependent upon the fusion of compatible mating types. This phenomenon is known as heterothallism, and has been well documented for the species by Collins (1961, 1963), Dee (1967) and Henney (1967). Collins (1963) and Collins and Ling (1964) demonstrated that the mating type system in this species is controlled by multiple alleles at a single locus. To date there is no visible difference between the cells of different mating types; yet the fusion itself involves exclusive mating type compatibility and is a rapid process (Yemma and Therrien, 1974). This process undoubtedly involves the plasma membrane (Yemma and Selanik, 1978).

Before investigation into the precise mechanism of fusion can be undertaken, an analysis of the plasma membrane must be made. This task was facilitated when a method for the isolation of the plasma membrane was developed here at

Youngstown State using a modification of the dextran-polyethylene glycol aqueous two-phase polymer system (Yemma and Selanik, 1978). This isolation technique was found to be superior to the widely accepted technique of density gradient centrifugation as applied to this particular cell system.

In this study a general biochemical characterization of the plasma membrane was undertaken utilizing exclusively the haploid phase of Didymium iridis, i.e., the myxamoeba and swarmers. Both cell types are capable of encystment under environmental stress, thereby making the plasma membrane inaccessible by the isolation technique used. Thus, special care was taken to exclude any encysted cells.

The literature contains many well documented methods in general use for the biochemical characterization of cell membranes. The procedure of SDS-polyacrylamide gel electrophoresis is widely used for the dissolution and analysis of membrane proteins ranging from mammalian cell systems (Fairbanks, et al., 1971) to the more closely related cellular slime mold, Dictyostelium discoideum (Spudich and Clark, 1974), and was deemed suitable for the characterization of the membrane proteins in this study. It was found that the incorporation of the anionic detergent sodium dodecyl sulfate (SDS) into the gel, sample solution, and buffers maintains to a great degree, the solubility of the proteins and also dissolves the lipid-protein aggregates found in the membrane which is essential. The value of this method is increased by the demonstration by Shapiro, et al., (1967)

and Weber and Osborn (1969) that the distance of migration of many soluble proteins in SDS-polyacrylamide gel electrophoresis under reducing conditions correlates well with their molecular weight. The basis of the technique is the similar binding of SDS by a wide variety of proteins on a weight to weight basis (Pitt-Rivers and Impiombato, 1968; Reynolds and Tanford, 1970). Binding induces configurational changes in the proteins, leading to uniform rod-like conformation, the length of which varies directly with the molecular weight of the polypeptide chain. The charge that is contributed by the SDS also is directly proportional to the length of the polypeptide chain. As the distance of migration on the polyacrylamide gel during electrophoresis is a function of each of these parameters, a directly proportional relationship with molecular weight is obtained. However, if the disulfide bonds remain intact, the binding of SDS is significantly decreased (Pitt-Rivers and Impiombato, 1968) and a simple proportionality no longer exists (Fish, et al., 1970). It is therefore necessary to treat the protein sample with 2-mercaptoethanol which reduces the disulfide linkages, thus allowing maximal SDS binding capacity. The general simplicity of the procedure plus the added advantage of molecular weight determinations made SDS-polyacrylamide gel electrophoresis the method of choice concerning the organism studied in this investigation.

Analysis of the membrane lipids is rather complicated regarding Didymium iridis. Phospholipids, glycolipids,

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sterols and other lipids found in membranes are associated with proteins, and because water is involved in the bonding between the protein and the lipid, a dehydrating solvent such as methanol must be used to rupture the lipid-protein linkage. It was found that many commonly occurring lipids were not soluble in this type of polar solvent. Thus, it was necessary to include a less polar solvent such as petroleum ether, chloroform, or diethyl ether (Johnson, 1971). The most common solvent mixture and the one utilized in this study for lipid extraction is chloroform-methanol 2:1 (v/v) first proposed by Folch and his co-workers in the 1950's (Folch, et al., 1957). Polar-nonpolar solvent systems almost always denature the proteins producing a protein residue difficult if not impossible to resolubilize (Rouser, et al., 1963).

Once extracted, the lipids were separated into their major classes by column chromatography. Separation by this technique depends upon a reversible interaction between the molecules being separated and the solid support (e.g., silicic acid) as these molecules are carried in a suitable solvent, the eluant, through the column. The process of interaction may be adsorption (the most common), liquid-liquid partition, ion-exchange, complex formation, or molecular sieving (Davenport, 1971).

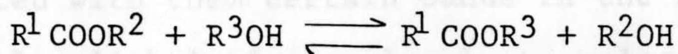
The order of elution and the degree of separation of the various lipid classes may be affected by conditions such as the activity of the silicic acid, the shape and

dimensions of the column, the method of preparing the column, the quality of the eluting solvents, the rate of elution, the temperature, and the fatty acid composition of the lipid (Davenport, 1971). Generally speaking, the polarity of the solvent necessary to elute a given substance increases with the polarity of the substance. Thus, in this study, the neutral lipids are eluted with chloroform, the glycolipids with acetone, and the phospholipids with methanol (Wood, et al., 1970; Parkes and Thompson, 1970; Law and Snyder, 1974).

The lipid classes were further fractionated by thin layer chromatography. Silica gel is the most frequently used adsorbent for the TLC of lipids, while the selection of solvent systems for the development of the chromatograms is dependent upon the nature of the mixture to be separated. Neutral lipids, for example, are best separated by nonpolar solvents such as hexane, petroleum ethers and benzene while amphiphilic lipids require strongly polar solvents (Shenstone, 1971). Differing ratios of chloroform-methanol-water have been used by many investigators to resolve the phospholipids and glycolipids found in the membranes of many different cell systems (Parkes and Thompson, 1970; Wood, et al., 1970; Beining, et al., 1975; Getz, et al., 1970). The addition of methanol and water to the developing solvent results in the adsorption of the polar solvent onto the silica gel. Resolution then occurs by partition in addition to adsorption (Shenstone, 1971). Once the chromatogram is

developed, a non-specific spray reagent is applied to ensure that all components of the chromatogram are visualized. Specific sprays may be subsequently used to classify the lipids in each of the various spots. Details of this and other aspects of TLC may be found in the excellent review of Mangold (1961).

Thin layer chromatography is often coupled to gas-liquid chromatography via the chemical process of interesterification (McGinnis and Dugan, 1965). Silica gel scrapings from the thin-layer chromatograms are subjected to alcoholysis, resulting in the following reaction:



$$R^1COOR^2 + R^3OH \rightleftharpoons R^1COOR^3 + R^2OH$$

The resulting products are then analyzed by gas-liquid chromatography. This method was first developed by James and Martin (1952) for the separation of volatile fatty acids. Since that time, there has been a tremendous growth in the use of the method, especially in the field of lipid biochemistry. The technique involves separating volatile substances by passing them in a gas stream over a liquid stationary phase spread as a thin film over an inert solid. The basis of the separation is the differential partitioning of the substance between the gas and the liquid film (Johnson and Stocks, 1971). Bullock and Dawson (1976) used the method to study the sterol content of the Myxomycetes _Physarum polycephalum_ and _P. flavicommum_. Other investigators are finding increasing use of GLC especially in tumor cell research (Wood, et al., 1970; Friedberg and Halpert, 1978).

Infrared spectroscopy (IR) is yet another method applied in this study. The utility of infrared spectrophotometry originates in the fact that the energy absorbed in the infrared region depends upon such factors as the masses of atoms and the strengths of the bonds which hold them together. For example, a diatomic molecule such as HCl has only one mode of vibration, the stretching and shortening of the C-Cl bond. The resistance to stretching and hence the frequency of radiation required to excite the vibration is characteristic of the particular arrangement of atoms in the molecule. Most groups, such as C-H, O-H, C=O, etc., have associated with them certain bands in the infrared which vary only slightly from molecule to molecule. Such bands are called group frequencies (Day and Underwood, 1974). By using these and other characteristic group bands, it is possible to establish the type of compound present. Heftmann and his co-workers (1960) used an IR approach in their study of sterols isolated from *Dictyostelium discoideum*. By the application of this and other methods previously described, it is hoped that this initial characterization of the plasma membrane of *Didymium iridis* will provide some insight into the workings of the membrane and its associated phenomenon.

## MATERIALS AND METHODS

### Tissue Culture

An isolate of D. iridis designated as Honduran a-2A[1] was used exclusively in this investigation. The culture originally was made available by Dr. O.R. Collins, Department of Botany, University of California, Berkeley.

### Growth of D. iridis on Solid Media

Clones of myxamoebae and swarmers were initially developed by single spore isolates of the Honduran 1-2A[1] mating type and maintained on slants of half strength 2% corn meal agar. The slants were previously innoculated with Escherichia coli to provide a food source for the cells. Plates containing sterile medium were streaked with approximately 1 ml of an E. coli suspension which developed into an even, bacterial lawn within 1 to 2 days. Transfers of the amoebae from slants to these plates were then made. In order to maintain the cells, subcultures were made by removing small pieces of agar from plates in log phase to new plates every 5 to 7 days. They were kept in a 23 degree C incubator (Yemma and Therrien, 1972; Yemma et al., 1974). Care was taken to exercise sterile technique throughout the transferring procedure, as cultures can easily become contaminated with other micro-organisms. Cultures were examined under the microscope periodically so that any contaminated ones might be discarded.

The medium used for both plates and slants was made by combining 8.5 grams of Difco Corn Meal Agar and 8.0 grams of Difco BactoAgar in 1 liter of distilled water. The mixture was them autoclaved and dispensed in 10 ml aliquots to plates (Collins, 1963; Yemma et al., 1974).

Growth of D. iridis in Liquid Culture

D. iridis was also grown in liquid culture in the following manner. Ten to twenty plates of cells in log phase were washed with .25M sucrose-phosphate buffer. The washings were pooled and centrifuged at 70 x gravity for 15 minutes in an Adams Dynac swinging bucket centrifuge. The pellet of cells was washed with .25M sucrose-phosphate buffer and re-centrifuged. Supernatant, consisting almost entirely of bacteria was discarded. The washings were repeated until the concentration of amoebae were from $5 \times 10^4$ to $1 \times 10^6$ cells/ml and bacteria numbered from $1 \times 10^9$ to $1 \times 10^{10}$/ml. The cells were resuspended in 2 to 4 ml of buffer between washings and counts were made using a hemocytometer (Yemma and Selanik, 1978). Extreme caution was used in observing sterile technique throughtout this procedure as air-borne yeast infections were a common problem.

The liquid medium for the cells was made by combining 10 grams of corn meal agar with .25M sucrose-phosphate buffer. This mixture was permitted to stir on an automatic stir plate for at least ½ hour. The undissolved agar was then filtered out of the solution through

Whatman No. 1 qualitative filter paper. The volume was then brought up to 1 liter with additional buffer, and autoclaved. It was occasionally necessary to refilter after autoclaving. After sterilization, the medium was permitted to cool to 23 degrees C and was them innoculated with the cells. The culture was placed on a reciprocating shaker bath maintained at 23 degrees C for one day. The amobae were then transferred to .25M sucrose-phosphate buffer: cell counts were made every two days and when the density reached approximately 1 x $10^6$ cells/ml, the culture was harvested and the cells were sterile (Yemma & Selanik, 1978).

The phosphate buffer used throughout this study was prepared as follows: Three grams of potassium chloride, 2.32 grams of sodium chloride, 9.04 grams sodium phosphate, dibasic and 18.4 grams potassium phosphate, monobasic were added to 4 liters of distilled water. This produced a 50mM phosphate buffer at pH 6.5.

## Harvest of Cells

Cells may be harvested from liquid and/or solid culture in similar ways. In either case, cells must be promptly harvested when log phase is attained. If there is delay after this point, encystment is likely to occur and the plasma membrane of the cell becomes inaccessible.

A yield of 0.5 ml packed volume of cells was obtained by washing 60 to 70 plates in log phase. Plates were flooded with .25M sucrose-phosphate buffer (pH 6.5) until the surface was just covered. Many of the cells will convert

to swarmers, and these were easily suctioned off the surface. In cases where a great number of myxamoebae adhered to the surface of the agar, they were gently washed with a Pasteur pipette using additional buffer. All washings were collected and spun down in 50 ml conical centrifuge (Yemma, 1971). The supernatant which consists almost entirely of bacteria was discarded. Cells in liquid culture are spun down in the same manner. Whether one chooses to grow cells in liquid culture or on plates is determined by the number of cells needed for experimental purposes. Liquid culture precludes the washing of a great number of plates and provides a large number of cells; however, it requires a greater number of centrifugations to obtain a pellet of cells. Both culturing techniques require about the same amount of time for harvesting the cells.

From this point on, treatment of the harvested cells is the same, whether they originated from plates or liquid culture. Although much of the bacteria has been discarded with the supernatant, there is some that remains in the pellet of cells. It has been found that if the pellet is re-suspended in 2 ml of 10 percent sucrose-phosphate buffer (w/v) and layered over 2 ml of 30 percent sucrose-phosphate buffer (w/v) and centrifuged at 42 x gravity for 25 minutes, the bacteria appears as a white band in the 10 percent sucrose layer. This procedure is repeated (usually 3 to 5 times) until the white band of E. coli is no longer visible. When the cells were determined microscopically to be amost free of bacteria, they were washed one more

time in .25M sucrose-phosphate buffer and centrifuged at 70 x gravity for 5 minutes. It has been noted that the repeated centrifugations tend to cause the swarmers to "round" up and most of the cells present at this point are myxamoebae. If the cells have a great number of bacterial adhering to their surfaces they were resuspended in a small amount of .25M buffer and placed on a reciprocating shaker bath for 12 to 24 hours. This allows the amoebae to ingest and digest the remaining bacteria. It is essential not ot let the cells go beyond this time, as starvation will cause encystment. Finally, the cells may be collected and homogenized in preparation for isolation of their membranes.

Homogenization of Cells + Isolation of Plasma Membrane

From this point on, all steps in the isolation procedure were carried out at 0 to 4 degrees C. The bright yellow pellet of amoebae was suspended in 2 to 4 ml of buffer and homogenized with 20 to 40 strokes of a Dounce homogenizer. The homogenization process was monitored microscopically. When it appeared that greater than 90% of the cells were broken the homogenization was stopped. The cell homogenate was then centrifuged for 15 minutes at 1400 revolutions per minute in a Damon IEC B-20A centrifuge. The pellet was placed into the two-phase polymer system for the separation and isolation of the plasma membrane according to the method of Yemma and Selanik (1978). (See Figure 1).

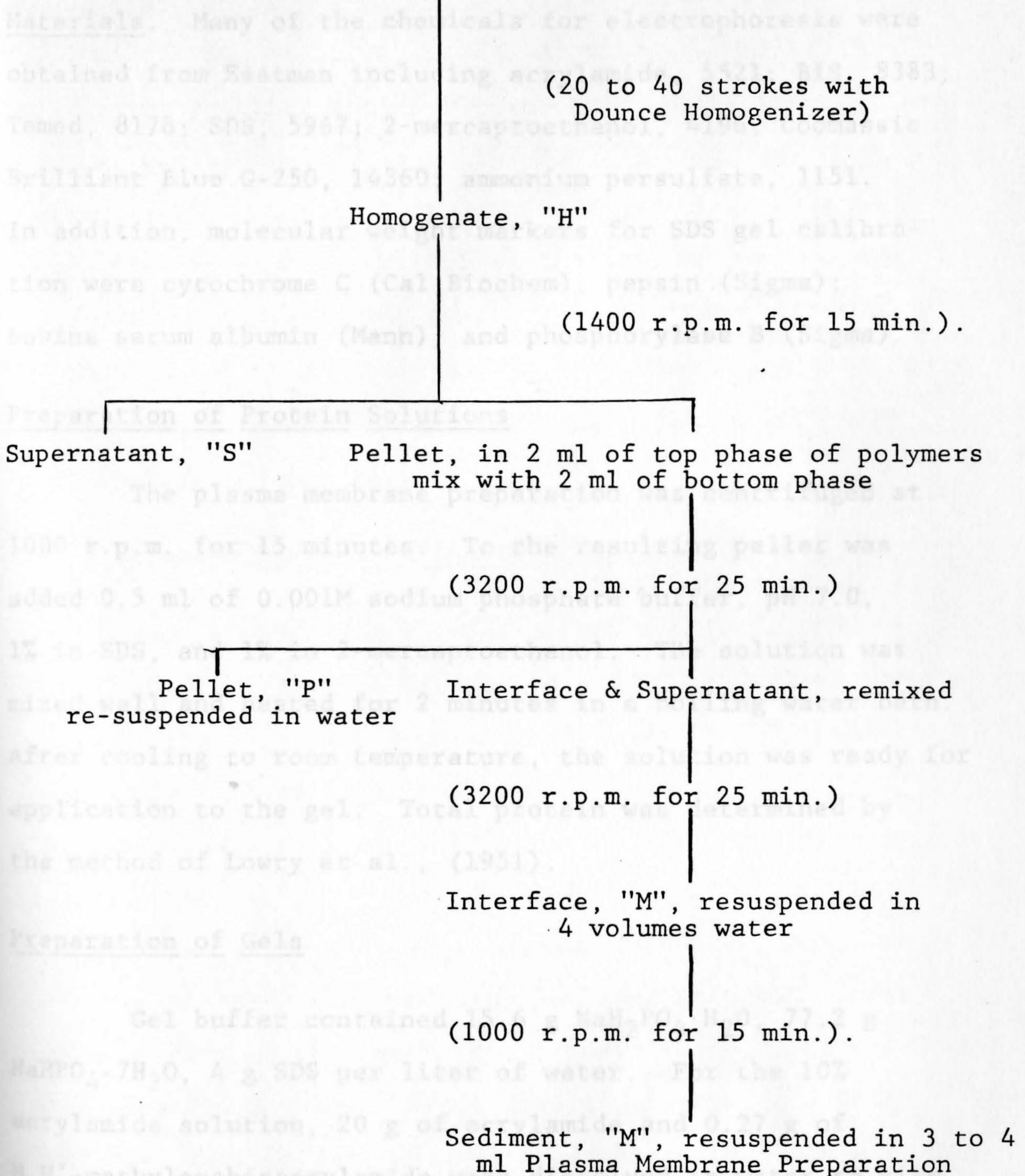



Fig. 1.--Summary of two-phase polymer isolation of membranes. (Yemma and Selanik, 1978).

## Membrane Proteins

Materials. Many of the chemicals for electrophoresis were obtained from Eastman including acrylamide, 5521; BIS, 8383; Temed, 8178; SDS, 5967; 2-mercaptoethanol, 4196; Coomassie Brilliant Blue G-250, 14360; ammonium persulfate, 1151. In addition, molecular weight markers for SDS gel calibration were cytochrome C (Cal Biochem); pepsin (Sigma); bovine serum albumin (Mann); and phosphorylase B (Sigma).

### Preparation of Protein Solutions

The plasma membrane preparation was centrifuged at 1000 r.p.m. for 15 minutes. To the resulting pellet was added 0.5 ml of 0.001M sodium phosphate buffer, pH 7.0, 1% in SDS, and 1% in 2-mercaptoethanol. The solution was mixed well and heated for 2 minutes in a boiling water bath. After cooling to room temperature, the solution was ready for application to the gel. Total protein was determined by the method of Lowry et al., (1951).

### Preparation of Gels

Gel buffer contained 15.6 g $NaH_2PO_4 \cdot H_2O$, 77.2 g $NaHPO_4 \cdot 7H_2O$, 4 g SDS per liter of water. For the 10% acrylamide solution, 20 g of acrylamide and 0.27 g of N,N'-methylenebisacrylamide were dissolved in water to give 50 ml of solution which was kept in the dark at 4 degrees D. A TEMED stock solution was prepared by diluting 0.3 ml of TEMED to 50 ml.

The glass gel tubes were 12 cm in length with an inner diameter of 6 mm. For a typical run of ten gels, 10.0 ml of the gel buffer was mixed with 10.0 ml of the acrylamide solution and 10.0 ml of the TEMED stock solution. To this was added 10.0 ml of the freshly prepared ammonium persulfate solution (3 mg/ml). After mixing, each tube was filled to within one inch of the top. A few drops of water were layered on top of the gel solution. After about 20 minutes, an interface could be seen indicating that the gel had solidified. The gels were then placed into the electrophoretic apparatus.

Preparation of Samples + Electrophoresis

For each gel, 3 ul of tracking dye (0.01% Bromophenol blue), 1 crystal of sucrose and 100-150 ul of the unknown protein solution or the molecular weight standards were mixed on a spotting dish and applied to the gels. The two compartments of the electrophoresis apparatus were filled with gel buffer, diluted 1:4 with water. Electrophoresis was performed at a constant current of 8 ma per gel with the positive electrode in the lower chamber. Under these conditions the tracking dye moved through the gel in approximately 8 hours. After electrophoresis, the gels were removed from the tubes by squirting water from a syringe between gel and glass wall. The length of the gel and the distance moved by the tracking dye were measured.

Staining and Destaining

The staining solution was prepared by dissolving 1.25 g of Coomassie brilliant blue in a mixture of 454 ml of 50% methanol and 46 ml of glacial acetic acid, and removing insoluble material through Whatman No. 1 filter paper. Staining was at room temperature for 30-60 minutes. The gels were removed from the staining solution, rinsed with distilled water and placed in a destaining solution consisting of 46 ml of glacial acetic acid and 227 ml of methanol diluted to 500 ml with water. Several changes were required over several days to completely destain the gels.

The gels swell some 5% in the acidic solution used for staining and destaining. Gels with a low amount of cross-linker show more swelling. Therefore, the calculation of the mobility has to include the length of the gel before and after staining as well as the mobility of the protein and of the marker dye. Assuming even swelling of the gels, the mobility was calculated as

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}$$

The mobilities were plotted against the known molecular weights expressed on a semi-logarithmic scale.

Other gels were stained for glycoproteins using the PAS procedure (Fairbanks, et al., 1971). Because high concentrations of SDS produced an intense background, the SDS was removed before PAS staining by carrying out the following steps: (1) 25% isopropyl alcohol, 10% acetic acid; overnight; (2) 10% isopropyl alcohol, 10% acetic acid; 6-9 hours; (3) 10% acetic acid; overnight; (4) 10% acetic acid; several hours. The fixed gels were then stained according to the following procedure: (1) 0.5% periodic acid ($H_5IO_6$); 2 hours; (2) 0.5% sodium arsenite, 5% acetic acid; 30-60 minutes; (3) 0.1% sodium arsenite, 5% acetic acid; 20 minutes - repeated twice; (4) acetic acid; 10-20 minutes; (5) Schiff reagent; overnight; (6) 0.1% sodium metabisulfite ($Na_2S_2O_5$), 0.01N HCl, for several hours, repeated until the rinse solution failed to turn pink upon the addition of formaldehyde.

Gels stained with Coomassie Blue were scanned on a Beckman densitometer at 595 nm, the maximum absorption for the stain.

## Membrane Lipids

### Lipid Extraction and Separation into Major Classes

The plasma membrane preparation was centrifuged at 1000 r.p.m. for 15 minutes to obtain a pellet. Lipids were extracted with 20 vol. of chloroform-methanol 2:1 (v/v) Folch, et al., (1957). To remove the precipitated protein, the extract was filtered through glass wool in

acid treated glass wool rinsed twice with 5.0 ml of extraction solvent. The lipids were separated into their major classes by column chromatography. The filtrate was placed on short columns of silicic acid - celite (acid washed) 2:1 (wt/wt). The neutral lipids were eluted with chloroform, the glycolipids with acetone, and the phospholipids with methanol. Each eluate was evaporated to dryness under a stream of $N_2$ in a water bath.

Neutral Lipids

The neutral lipids were redissolved in chloroform and ten microliters were carefully spotted on the center of a potassium bromide pellet with a microsyringe. An infrared spectrum was obtained using a Beckman 4200 dual beam infrared spectrophotometer. The neutral lipids were recovered from the KBr pellet with chloroform and spotted on thin layers of Silica gel G (Quantum Q5, 250 microns). One of the thin layer plates was developed to a height of 15 cm in petroleum ether-diethyl-ether-acetic (90:10:1), the other in hexane-diethyl ether-acetic acid (90:10:1). Spots were visualized by spraying with 0.001% aqueous Rhodamine 6G and viewing the damp plates under ultraviolet light. The spots were scraped from the plate with a razor blade and transferred to Pyrex test tubes where the silica gel scrapings were mixed with 2.0 ml of 6% $H_2SO_4$ in methanol (Feldman and Rouser, 1965). The tubes were sealed and heated at 110 degrees C for 2 hours.

The neutral lipid was extracted into hexane and taken to dryness under $N_2$. The lipids were redissolved in chloroform and a second infrared spectrum was obtained.

Chromatographic analyses of the hexane extract were made on a Bendix Toxichrom gas chromatograph. The chromatographic separations were performed on dual 3 foot glass columns packed with 3% OV-7. Temperature was programmed from 200-260 degrees C with temperature increasing at the rate of 10 degrees C/min. Retention times were compared to those of standards and with published values.

The remaining neutral lipid from the hexane extraction procedure was spotted on TLC plates and developed in chloroform to a height of 15cm. Spots were located by spraying with 50% $H_2SO_4$ and heating at 100 degrees C for 15 minutes (Bullock and Dawson, 1976). A blue spot at a $R_f$ of about .34 indicated the presence of free sterols. Since the sulfuric acid is destructive, it was necessary to develop two samples and spray the other sample with the nondestructive Rhodamine 6G so that the spot correlating to the free sterols might be recovered. The sterols were extracted from the silica gel scraping with chloroform and analyzed by gas chromatography as described above.

A gas chromatograph was also obtained of the neutral lipid immediately after column chromatography and at the time of the first infrared spectrum so that this data might be compared to that obtained after TLC and interesterification.

Phospholipids

The phospholipids were redissolved in methanol spotted on thin-layers of Silica gel G and developed in chloroform-methanol-water (65:25:4) to a height of 13 cm. Blue spots were detected after spraying with Phospray (Supelco), a specific spray sensitive to about one microgram of phosphorous. The $R_f$ of each spot was calculated and compared to standards and with published values.

Glycolipids

The glycolipids were redissolved in acetone and spotted on thin layers of Silica gel G. The chromatogram was developed in chloroform-methanol-water (65:25:4). Spots were visualized with Diphenylamine(Supelco), a specific spray for glycolipids.

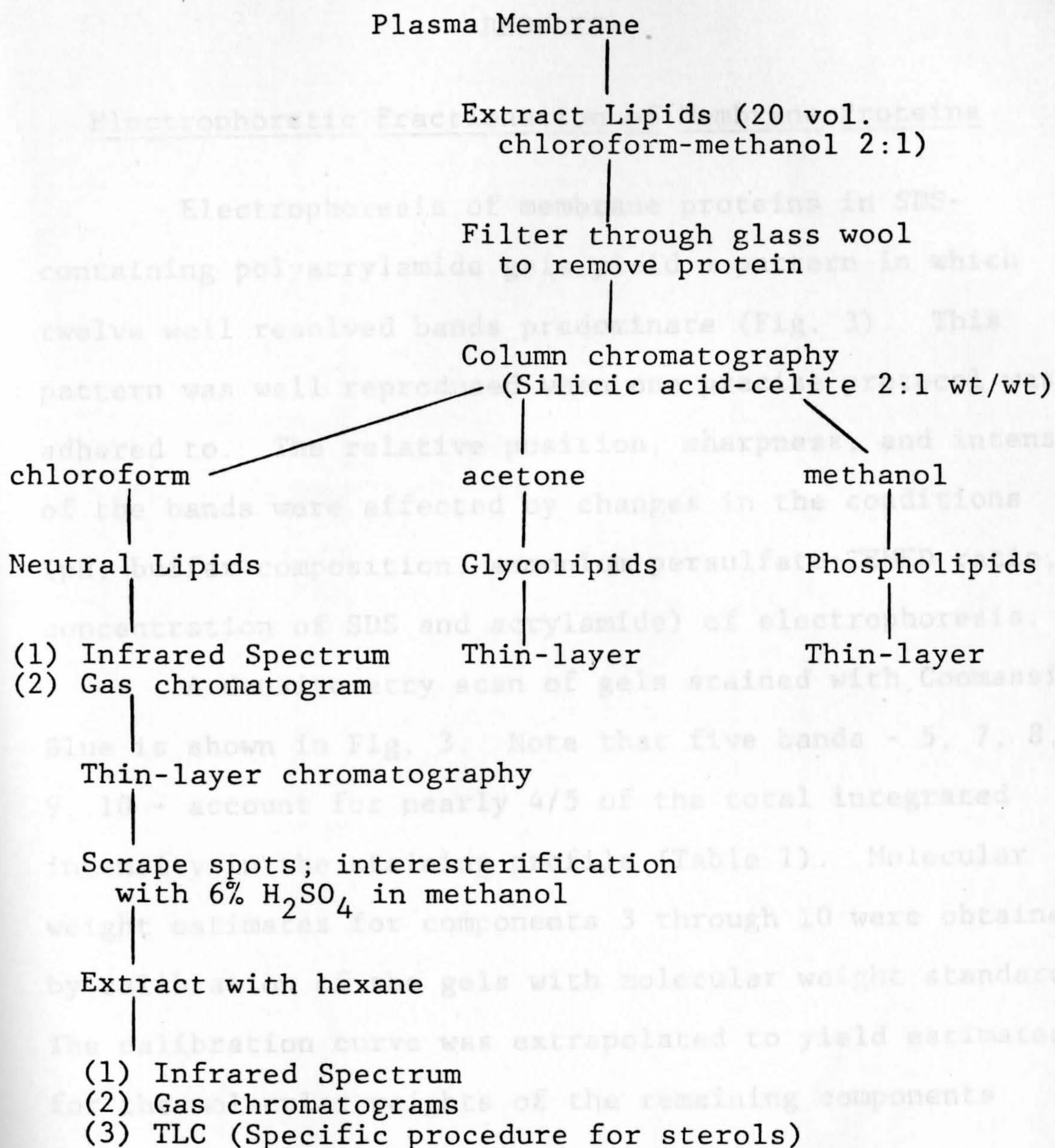



Fig. 2. --Summary of Lipid Methodology

# RESULTS

## Electrophoretic Fractionation of Membrane Proteins

Electrophoresis of membrane proteins in SDS-containing polyacrylamide gels yield a pattern in which twelve well resolved bands predominate (Fig. 3). This pattern was well reproduced when one precise protocol was adhered to. The relative position, sharpness, and intensity of the bands were affected by changes in the conditions (pH, buffer composition, ammonium persulfate:TEMED ratio, concentration of SDS and acrylamide) of electrophoresis.

A densitometry scan of gels stained with Coomassie Blue is shown in Fig. 3. Note that five bands - 5, 7, 8, 9, 10 - account for nearly 4/5 of the total integrated intensity in the staining profile (Table 1). Molecular weight estimates for components 3 through 10 were obtained by calibration of the gels with molecular weight standards. The calibration curve was extrapolated to yield estimates for the molecular weights of the remaining components (Fig. 4) (Table 1).

When stained for carbohydrate by the PAS procedure, two PAS-positive components are resolved corresponding to the two slowest moving bands on the gels stained with Coomassie Blue. The molecular weights are very large with estimates in the range of 200,000. The low mobilities, in addition to the PAS-positive activity suggest very strongly that the two PAS-positive components are glycoproteins.

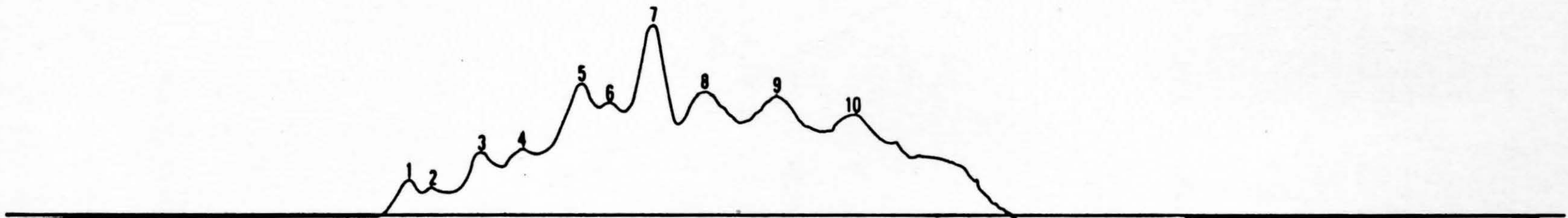
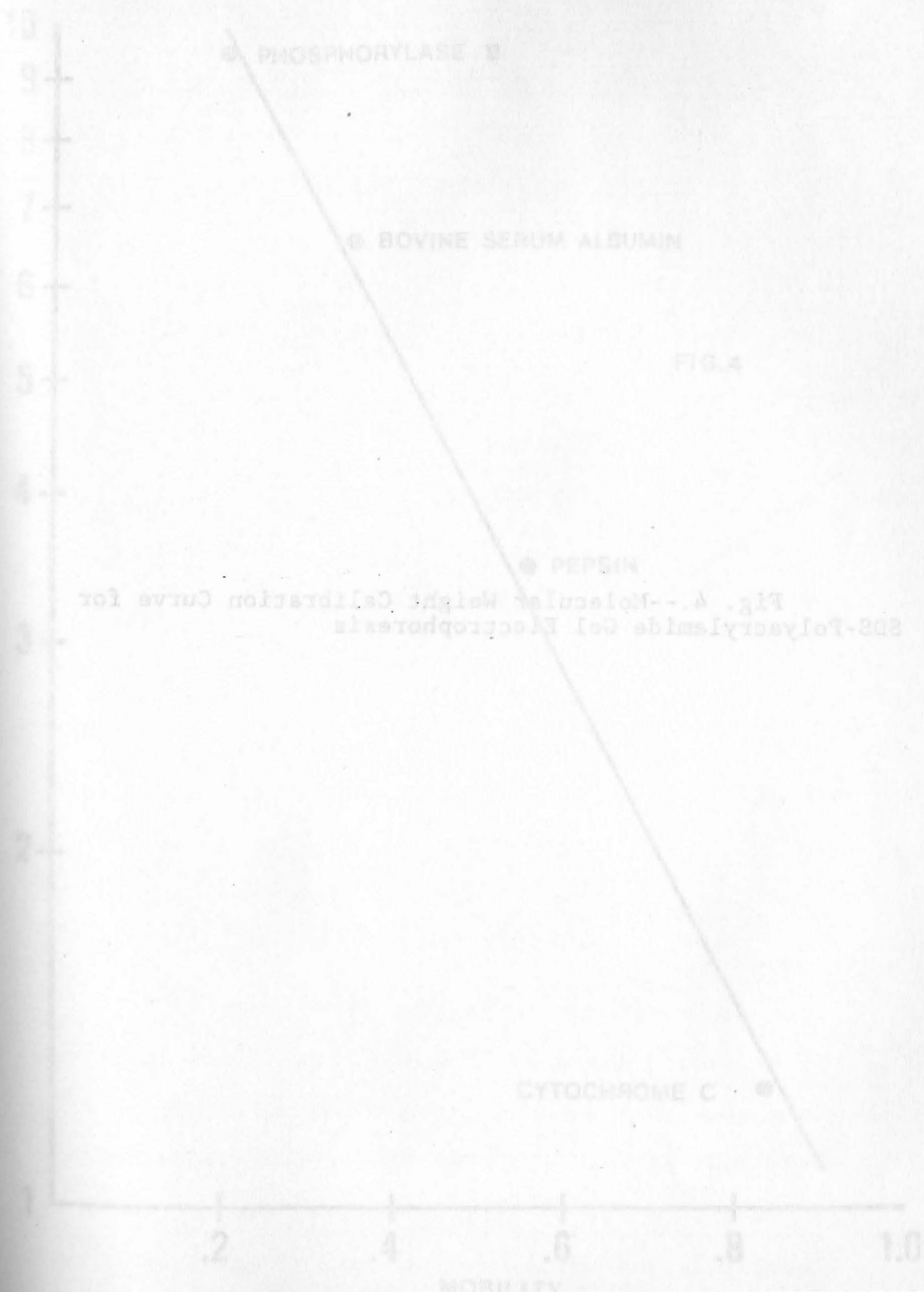
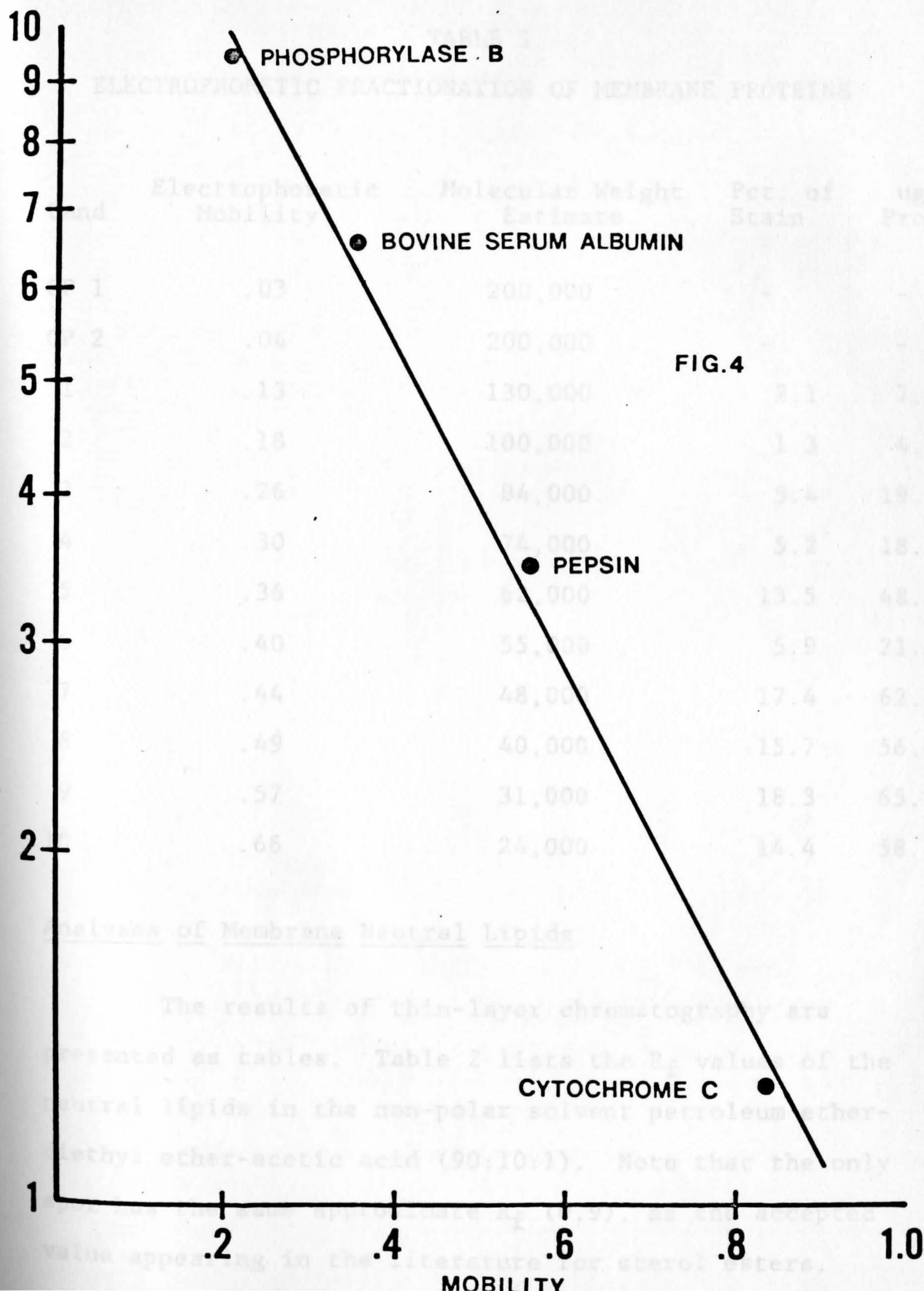



Fig. 3.--Gel Electrophoresis Pattern and Densitometer Tracing of Membrane Proteins.


PHOSPHORYLASE B
BOVINE SERUM ALBUMIN
FIG.4
PEPSIN
CYTOCHROME C
10
9
8
7
6
5
4
3
2
1
.2
.4
.6
.8
1.0
MOBILITY



10
9
8
7
6
5
4
3
2
1
MW
PHOSPHORYLASE B
BOVINE SERUM ALBUMIN
PEPSIN
CYTOCHROME C
FIG.4
.2
.4
.6
.8
1.0
MOBILITY

They are appropriately designated as GP 1 and GP 2 (Fig. 3) (Table 1).

TABLE 1

ELECTROPHORETIC FRACTIONATION OF MEMBRANE PROTEINS

| Band | Electrophoretic Mobility | Molecular Weight Estimate | Pct. of Stain | ug Prot. |
|---|---|---|---|---|
| GP 1 | .03 | 200,000 | - | - |
| GP 2 | .04 | 200,000 | - | - |
| 1 | .13 | 130,000 | 2.1 | 7.6 |
| 2 | .18 | 100,000 | 1.3 | 4.7 |
| 3 | .26 | 84,000 | 5.4 | 19.4 |
| 4 | .30 | 74,000 | 5.2 | 18.7 |
| 5 | .36 | 62,000 | 13.5 | 48.6 |
| 6 | .40 | 55,000 | 5.9 | 21.2 |
| 7 | .44 | 48,000 | 17.4 | 62.6 |
| 8 | .49 | 40,000 | 15.7 | 56.5 |
| 9 | .57 | 31,000 | 18.3 | 65.8 |
| 10 | .66 | 24,000 | 14.4 | 58.8 |

Analyses of Membrane Neutral Lipids

The results of thin-layer chromatography are presented as tables. Table 2 lists the $R_f$ values of the neutral lipids in the non-polar solvent petroleum ether-diethyl ether-acetic acid (90:10:1). Note that the only spot has the same approximate $R_f$ (0.9), as the accepted value appearing in the literature for sterol esters.

The same neutral lipid is resolved into four components when the petroleum ether is replaced in the developing solvent by hexane (Table 3).

TABLE 2

TLC OF LIPIDS[a]

| Lipid | Approximate $R_f$ Value |
|---|---|
| Hydrocarbons | 1.0 |
| Sterol esters | 0.9 |
| Esters of long chain alcohols and acids | |
| Diacylglycerol monoethers | 0.7 |
| Methyl esters; increasing insaturation and decreasing chain length give lower $R_f$ values | |
| Aldehydes | |
| Triacylglycerols | 0.5 |
| Fatty acids (not ionized) | 0.25 |
| Alcohols | |
| 1,3-Diacylglycerols | |
| 1.2-Diacylglycerols | |
| Sterols | |
| Fatty acids (ionized) | |
| Monoacylglycerols | |
| Phospholipids | Remain at origin |
| Glycolipids | |
| Membrane neutral lipid | 0.9 |
| Standards: | |
| Cholesterol | .21 |
| Ergasterol | .18 |

[a]Silica gel G activated at 110° C, developed with petroleum ether-diethyl ether-acetic acid, 90:10:1 by volume, to 15 cm in a lined tank. (Johnson and Davenport, 1971).

TABLE 3

TLC OF NEUTRAL LIPID[a]

| Lipid | Approximate $R_f$ Value |
| --- | --- |
| Membrane Neutral Lipids: | |
| Neutral Lipid 1 | 0.06 |
| Neutral Lipid 2 | 0.28 |
| Neutral Lipid 3 | 0.52 |
| Neutral Lipid 4 | 0.61 |

[a]Silica gel G developed with hexane-diethyl ether-acetic acid 90:10:1 by volume, to 15 cm in a lined tank.

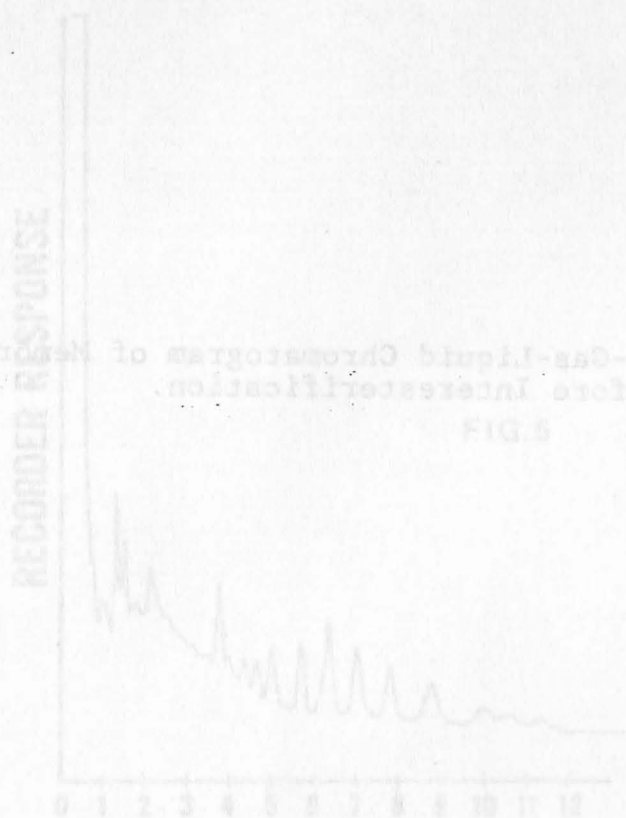
The neutral lipid was analyzed before and after interesterification by gas-liquid chromatography (GLC) as well as infrared spectroscopy (IR). The results of the GLC are presented in Figures 5 and 6. Note in Fig. 5 the abundance of earlier resolved peaks (between 0-4 min.) which are removed by the interesterification reaction (fig. 6). Note also the length of the retention times which are indicative of large molecular structures. The last peak has a retention time of just over twelve minutes, indicating molecules which are much larger than fatty acids (methyl stearate, RT = 1.0 min.) and triglycerides (tripalmitin, RT = 1.6 min.). When sterol standards were analyzed, the retention times (cholesterol, 9.1; ergasterol, 10.2) were much more compatible with those of the membrane neutral lipid fraction. The gas-liquid chromatogram represented in Fig. 6 was identical for each of the four

spots scraped from the thin-layer. Thus, the resulting product of the interesterification reaction is the same for each spot and the different $R_f$ values (Table 3) are due to different groups to which the product is attached.

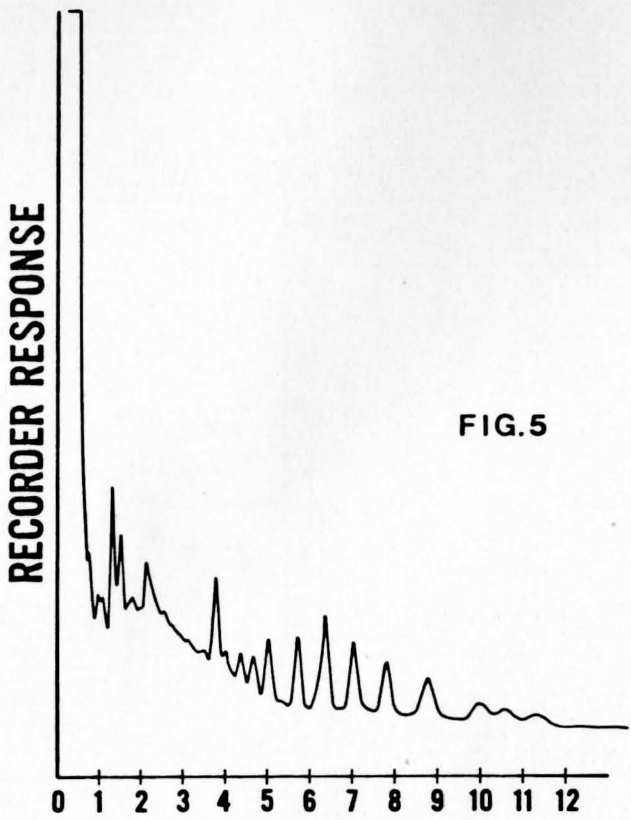
The infrared spectrum of the neutral lipid component before interesterification is shown in Fig. 7. The intense absorption band at $1740^{cm-1}$ is characteristic of the very strong C=0 stretch of esters. Because the C=0 stretch of other groups absorb at different wavelengths (e.g. carboxyl of free fatty acid, $1710^{cm-1}$; aldehydes and ketones, $1650\text{-}1800^{cm-1}$), it seems reasonable to assume that the membrane neutral lipid is present as an ester.

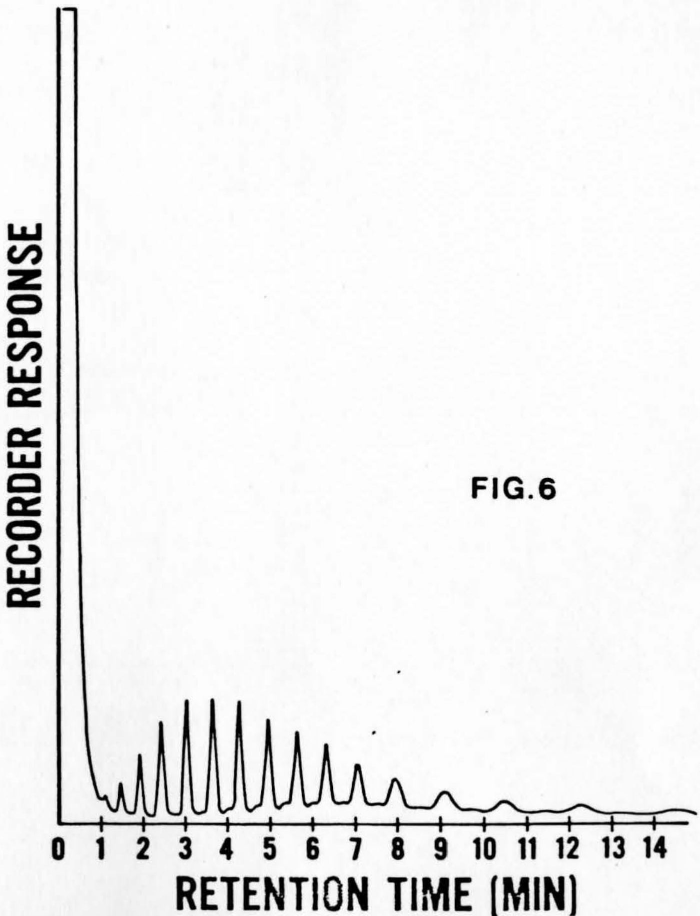
After interesterification (Fig. 8), the C=0 stretch at $1740^{cm-1}$ is absent and the overall spectrum generally resembles that of a sterol (Pouchert, 1970). If the molecules in question are indeed sterol esters, it appears that the ester linkage is being broken during the interesterification reaction resulting in free sterols.

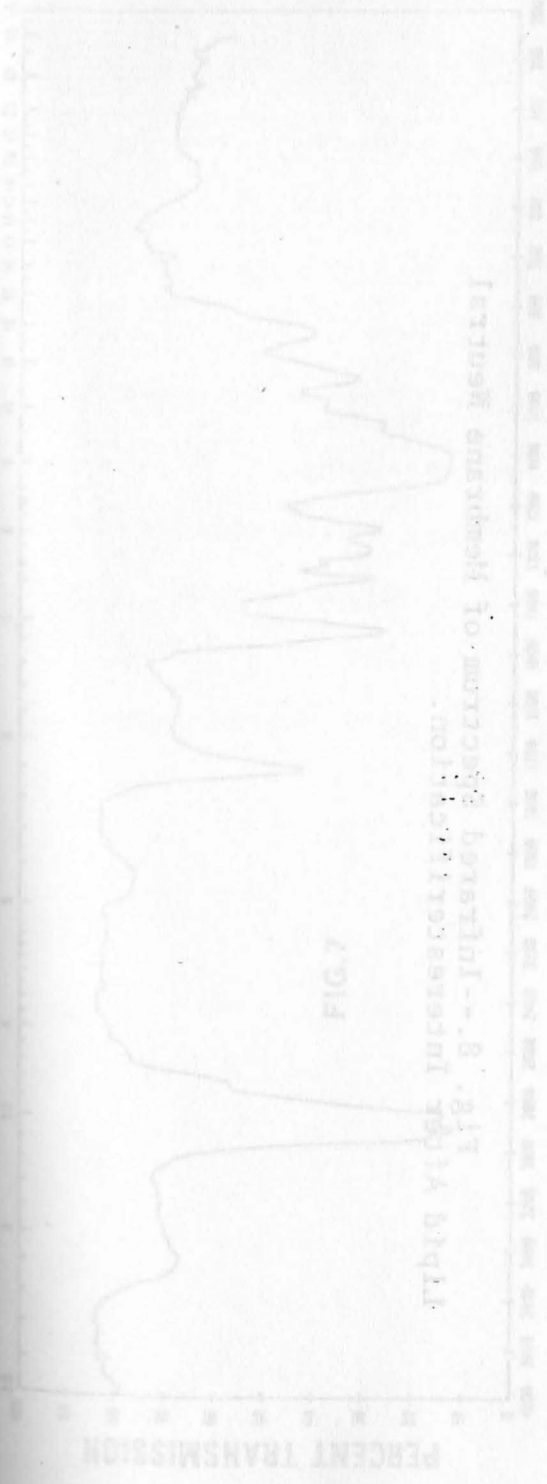
The membrane neutral lipid was subjected to a final TLC procedure. The membrane fraction after interesterification was developed in chloroform and sprayed with 50% $H_2SO_4$ - a specific test for free sterols (Bullock and Dawson, 1976). The resulting blue spot at an $R_f$ of 0.3 correlates well with the published value and confirms the presence of sterols in the neutral lipid fraction. When this spot was scraped from the thin-layer for GLC analysis, the resulting profile was again identical to the chromatogram


RECORDER RESPONSE
FIG.5
0 1 2 3 4 5 6 7 8 9 10 11 12

RECORDER RESPONSE
FIG.6
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
RETENTION TIME (MIN)


RECORDER RESPONSE
FIG.5
0 1 2 3 4 5 6 7 8 9 10 11 12


RECORDER RESPONSE
FIG.6
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
RETENTION TIME (MIN)



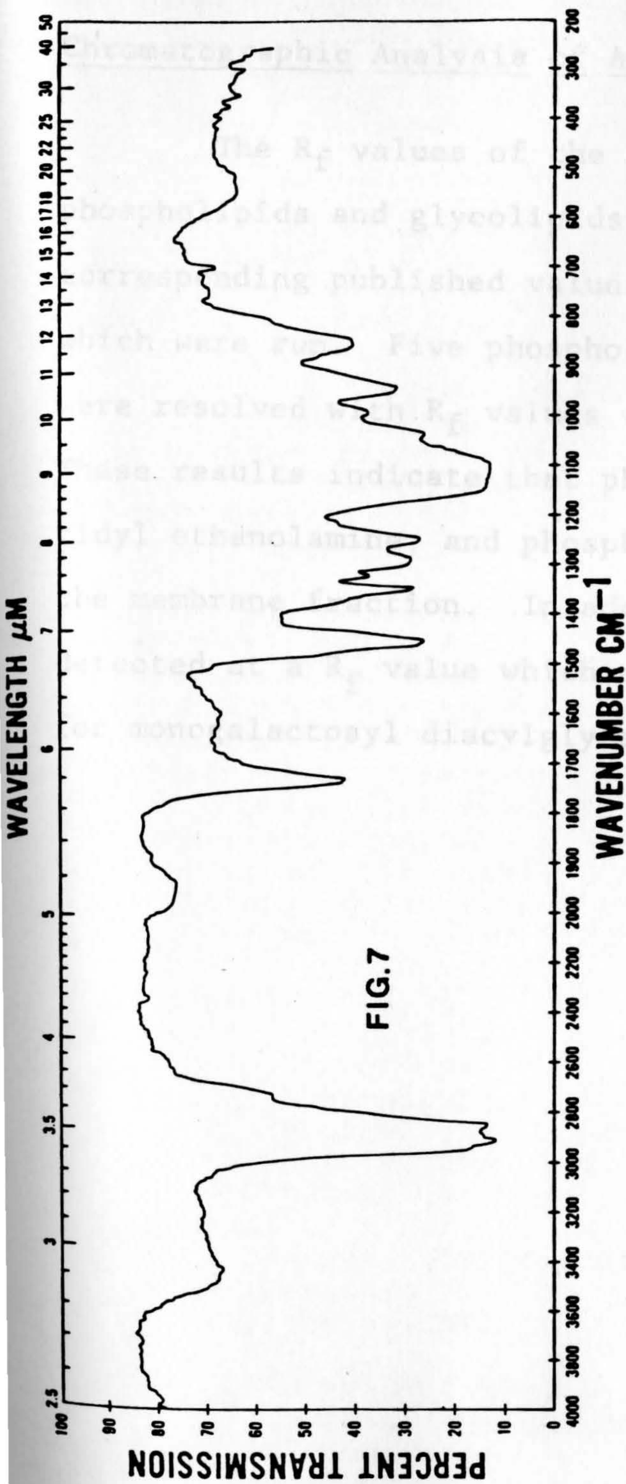



FIG.7

FIG.8

in Fig. 6. These results strongly indicate that the 14 components represented in that profile (Fig. 6) are sterols.

Chromatographic Analysis of Amphiphilic Lipids

The $R_f$ values of the amphiphilic lipids, i.e., phospholipids and glycolipids, are listed in Table 4 with corresponding published values as well as various standards which were run. Five phospholipids, labeled A through E, were resolved with $R_f$ values ranging from 0.07 to 0.42. These results indicate that phosphatidyl inositol, phosphatidyl ethanolamine, and phosphatidyl choline are present in the membrane fraction. In addition one glycolipid was detected at a $R_f$ value which agrees with the published value for monogalactosyl diacylglycerol.

TABLE 4

TLC OF AMPHIPHILIC LIPIDS[a]

| Lipid | Approximate $R_f$ Values[b] |
|---|---|
| Diphosphotidylglycerol | |
| Phosphatidic acid | |
| Monogalactosyldiacylglycerol | |
| Ceramide-monohexosides | 0.6 |
| (cerebrosides), resolve into several spots of decreasing $R_f$ due to number of hydroxyl groups in the fatty acids | |
| Ethanolamine plasmalogen | |
| Phosphatidylethanolamine | 0.45 |
| Digalactosyldiacylglycerol | |
| Phosphatidylglycerol | |
| Sulfolipids | |
| Sulfatides, split into two spots | 0.3 |
| Choline plasmalogen | |
| Phosphatidyl choline | |
| Lysophosphatidylethanolamine | 0.2 |
| Phosphatidyl serine | |
| Sphingomyelin containing long chain acids | |
| Sphingomyelin containing short chain acids | |
| Phosphatidyl inositol | |
| Lysophosphatidylcholine | 0.05 |
| Lysophosphatidylserine | Remain at origin |
| Gangliosides | |
| | |
| Membrane glycolipid | 0.62 |
| Membrane Phospholipids: | |
| Phospholipid A | 0.07 |
| Phospholipid B | 0.15 |
| Phospholipid C | 0.19 |
| Phospholipid D | 0.37 |
| Phospholipid E | 0.42 |
| Standards: | |
| Phosphatidylethanolamine | 0.46 |
| Lecithin | 0.24 |
| Lysolecithin | 0.08 |

[a] Developed on silica gel with chloroform-methanol-water, 65:25:4 by volume, in a lined tank

[b] $R_f$ values will vary considerably according to layer activation and with minor changes in solvent ratios (Johnson and Davenport, 1971).

# DISCUSSION

Initially, fractionation of the membrane proteins was attempted using the nonionic detergent Trition X-100 as the solubilizing agent. Electrophoresis was carried out in a Tris-EDTA-borate buffer, pH 8.4. Although no electrophoretic pattern resulted, it appeared that the failure was due to the inability of the detergent to solubilize the membrane. It is commonly noted in the literature (Steck and Fox, 1974) that proteins do not readily penetrate polyacrylamide gels. This is usually related to the fact that the proteins are not completely dispersed but are present as complexes or aggreates. In addition, some solubilized proteins may reaggregate upon exposure to the nearly neutral buffers of high ionic strength usually used in electrophoresis (Steck and Fox, 1974).

Subsequent attempts to solubilize the membrane extract utilized a mixture of phenol-acetic acid-water 2:1:1 (w/v/v), made 2M in urea, as the sample solvent. The approach was a modification of the procedure of Takayama and his coworkers (1964), developed specifically for the analysis of mitochondrial proteins. The presence of acetic acid in this system served several purposed: 1) it facilitated the solubilization of proteins in phenol, 2) it enhanced electrophoretic mobility by conferring a strong positive charge on the proteins and 3) it suppressed the accumulation of cyanate, which is formed by the decomposition of urea

(Fox, 1974). However, this procedure also met with little success, which was largely due to the fact that this experimental system was originally developed for the analysis of lipid-free proteins.

Solubilization of the membrane proteins and subsequent electrophoresis was finally accomplished using the SDS-PAGE system previously described (Weber & Osborn, 1969). Although SDS-electrophoresis is the most widely used method for the fractionation of membrane proteins, several problems can occur and should be kept in mind when an analysis of the results is being made. One is aggregation. In an ideal case, proteins are totally dissociated in SDS prior to electrophoresis. In practice, multichain complexes may persist under these conditions. The complexes may appear as discrete bands or as diffuse zones (Steck & Fox, 1974).

Another issue is that of proteolysis. When SDS is introduced into a supposedly purified membrane sample, the membrane proteins unfold and become many more times sensitive to the actions of proteases, which may be released from latency in the lysosomes at the same time (Steck & Fox, 1974). It is known that at least some proteases appear to withstand SDS denaturation long enough to cause extensive digestion of the vulnerable membrane proteins (Steck, et al., 1971). However, assays for the purity of our membrane reveal that very little contamination by the lysosomes occurs (Yemma and Selanik, 1978). Thus, proteolysis artifacts did not interfere in this study.

The electrophoretic profile presented

possibility must be considered that all twelve are not distinct proteins. It is possible that bands 8, 9 and 10, which are present as diffuse zones represent protein aggregates (doublets, triplets, etc.) while other, more clearly distinct bands are representative of polypeptide subunits. The heaviest bands, are very likely glycoproteins as they have the same electrophoretic mobilities as the PAS-positive components stained on corresponding gels. Further evidence of the presence of glycoproteins in these bands is the fact that Commassie brilliant blue was not always readily adsorbed by these bands. It is presumed that the heavy coat of oligosaccharides, composing 50% or more of the glycoprotein mass blocks its binding. Therefore, it was necessary to use the periodic acid-Schiff (PAS) procedure for carbohydrates to stain for the glycoproteins (Fairbanks, et al., 1971; Holden, et al., 1971). The method is difficult to quantitate since the color intensity which develops is somewhat evanescent and sugar dependent. But because the PAS-positive components had relative electrophoretic mobilities corresponding to bands GP1 and GP2, it is assumed that they are glycoproteins capable of adsorbing Coomassie blue.

Molecular weight estimates for bands 3 - 10 are said to have an accuracy of better than ± 10% (Shapiro, et al., 1967). But due to the lack of available standards in the range of 100,000 - 200,000, estimates were made for the remaining components. Previously, Weber and Osborn (1969) had found that use of a low proportion of cross-linking agent in their 10% acrylamide gels (i.e. 1.35g N,N

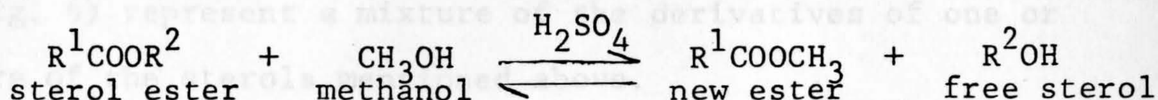
methylenebisacrylamide) resulted in a concave log MW mobility curve. Although our experiment used cross-linker in the same proportion, the curve remained linear throughout, thus ensuring reliable molecular weight determinations.

It is important to note that the membrane profile presented in Figure 3 was reproducible when the same protocol was adhered to. Also that none of the banding pattern can be attributed to bacterial contamination as bacterial controls were consistently negative.

The membrane protein pattern of the Myxomycete Dictyostelium discoideum has been presented in the literature (Spudick and Clark, 1974). But it is difficult to critically compare the results with that of our organism for several reasons. The Dictyostelium membranes were isolated using a sucrose gradient, solubilized in Triton X-100, and electrophoreses under different conditions. Although the protocols were vastly different, the same approximate number of components were resolved.

The neutral lipid fraction underwent analysis following the general approach of Parkes and Thompson (1970) with several modifications. The results of the TLC in petroleum ether-diethyl ether-acetic acid (90:10:1) revealed that the only spot resolved corresponded to the published value for sterol esters. When petroleum ether was replaced by hexane in the developing solvent, the same fraction was separated into four spots. When these spots were scraped, subjected to interesterification, and analyzed by GLC, the resulting chromatograms were identical for each of the four

spots (Fig. 6). This can easily be explained by close experiments of the interesterification reaction. It must be remembered that the thin-layer chromatography takes place before interesterification while the GLC profile is obtained subsequent to the reaction. The reaction being:



$$\underset{\text{sterol ester}}{R^1COOR^2} + \underset{\text{methanol}}{CH_3OH} \overset{H_2SO_4}{\rightleftharpoons} \underset{\text{new ester}}{R^1COOCH_3} + \underset{\text{free sterol}}{R^2OH}$$

It is assumed that the $R^1$ moeity to which the sterol is esterified is different for each of the four components thus resulting in varying migration distances on the TLC plates. Then prior to the GLC analysis, the free sterol, which is similar for all four spots, is extracted into the hexane while the new methyl ester is left behind in the interesterification reagent. That the hexane extract is, indeed, comprised of free sterols is further confirmed by a positive result in a TLC procedure specific for sterols.

Additional evidence is provided by the infrared data. Before interesterification (Fig. 7), an intense stretch bond of an ester is present. After the reaction, the band is absent (Fig. 8) and the spectrum resembles that of cholestanol, a compound whose derivatives have been found in two other species of Myxomycetes - Dictyostelium discoideum and Physarum polycephalum (Heftmann, et al., 1960). D. discoideum is also known to contain ergasterol and stigmasterol and their derivative, while in the plasmodia of P. polycephalum, lanosterol is suspected (Heftmann, et al., 1960; Bullock and Dawson, 1976).

Since the retention times in GLC will vary with very small changes in structure and with sterochemical and isomeric differences (Clayton, 1962; Blondin, et al., 1967; Ziffer, et al., 1960), it is suggested here that the 14 components resolved when the hexane extract is analyzed (Fig. 6) represent a mixture of the derivatives of one or more of the sterols mentioned above.

The phospholipid composition of the plasma membrane, as determined by thin-layer chromatography was essentially similar to that found in most biological membrane systems and was not unexpected. Although it is evident that additional biochemical analyses must be done on the plasma membrane of D. iridis it appears quite certain that phosphatidyl ethanolamine, phosphatidyl inositol, and phosphatidyl choline are present. These lipids as well as 2 other components not yet identified were detected using Phospray (Supelco), a specific spray for phosphorous-containing compounds sensitive to one microgram of phosphorous. It appears that this sensitivity was needed as continued attempts to scrape the spots for fatty acid analysis of the phosphatides met with failure. The small amount of phospholipid recovered from the membrane is rather surprising since phospholipids characteristically play a major role in the structure of most membrane systems. It is possible that the plasma membrane of this organism contains smaller amounts of phospholipids that are commonly present in some other organisms. Nevertheless, because of the sensitivity of the procedures used the five components were able to be resolved.

This study suggests that the biochemistry of the plasma membrane of the Myxomyces is highly complex. It is quite certain that many of the common molecular features of other plasma membrane systems are present. However, it is quite evident that in many respects the plasma membrane of this organism is unique, distinct and interesting.

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