A METHOD FOR ISOLATION OF THE PLASMA MEMBRANE OF THE MYXAMOEBAE AND SWARM CELLS OF THE MYXOMYCETE DIDYMIUM IRIDIS USING AN AQUEOUS TWO-PHASE POLYMER SYSTEM

by

Patricia E. Selanik

Submitted in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the

Biology

Program

Dr. John J. Jeanneau 8-12-76

Adviser Date

Dean of the Graduate School Aug. 16 1976

Date

Youngstown State University

August, 1976

ABSTRACT

A METHOD FOR ISOLATION OF THE PLASMA MEMBRANE OF THE MYXAMOEBAE AND SWARM CELLS OF THE MYXOMYCETE DIDYMIUM IRIDIS USING AN AQUEOUS TWO-PHASE POLYMER SYSTEM

Patricia E. Selanik

Master of Science

Youngstown State University, 1976

A method for the isolation of the plasma membranes of the acellular slime mold, Didymium iridis was developed using a modification of the dextran-polyethylene glycol aqueous two-phase polymer system. It was found to be superior to the widely accepted technique of density gradient centrifugation, as applied to this particular cell system. A number of chemical and enzymatic assays performed on the membrane and other cell fractions are discussed. These were used as a method for positive identification of the plasma membrane and assessment of its purity. The Feulgen reaction was used as an assay for nuclear contamination. Cytochrome oxidase, a mitochondrial marker, and acid phosphatase, a lysosomal marker, were used to check for contamination of the membrane fraction by those two sub-cellular organelles. The membrane preparation was also assayed for the presence of 5'-nucleotidase, an important and characteristic enzyme, present in most plasma membranes and widely used as a marker in its isolation and identification. All steps of the isolation procedure were monitored by phase-contrast and light microscopy.

Results of the chemical and enzymatic assays indicate that plasma membranes are recovered with high yield and purity using a modified

two-phase polymer technique. The method is not only rapid, but also can be performed using low-speed centrifugation equipment.

ACKNOWLEDGEMENTS

I wish to express sincere thanks to Dr. John J. Yemma, whose guidance and help made realization of this thesis possible. Special recognition belongs to Mr. Leonard Perry, staff microbiologist, and to the staff of the Northeastern Ohio Forensic Laboratory for their expert technical assistance and co-operation throughout the pursuit of this study.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF FIGURES

LIST OF TABLES

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 interesting 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). (See plate 1).

Generally, the development of this organism proceeds from a haploid or gametic stage in which it strongly resembles an animal form to zygote formation which through further development, results in a diploid plasmodium or vegetative stage. In the diploid stage the organism become increasingly plant-like in character, and after a period of migration, sporogenesis and spore formation takes place. Upon germination, spores produce either haploid myxamoeba or swarm cells (possessing two flagella), depending upon the cells' environment. The two types of cells are interconvertible; however, an aqueous environment favors the formation of swarmers. With bacteria as the major food source the population increases by mitotic divisions, and eventually log phase is attained. Some strains of myxomycetes are self-fertile or homothallic. In other strains, including the one used in this study, zygote formation and subsequent plasmodial formation is dependent upon the fusion of compatible mating types. This phenomenon is known as heterothallism, and has been well documented for the species

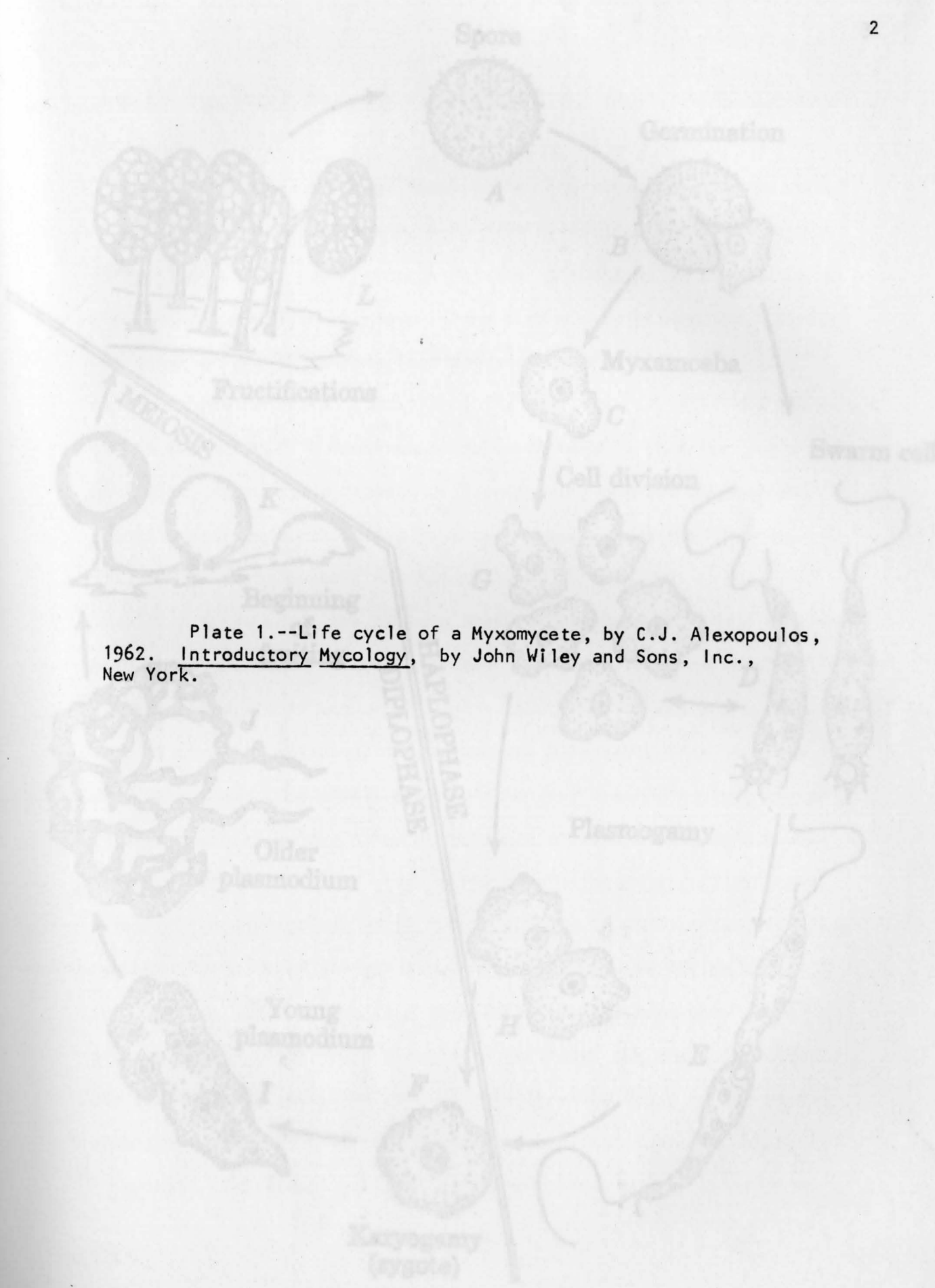
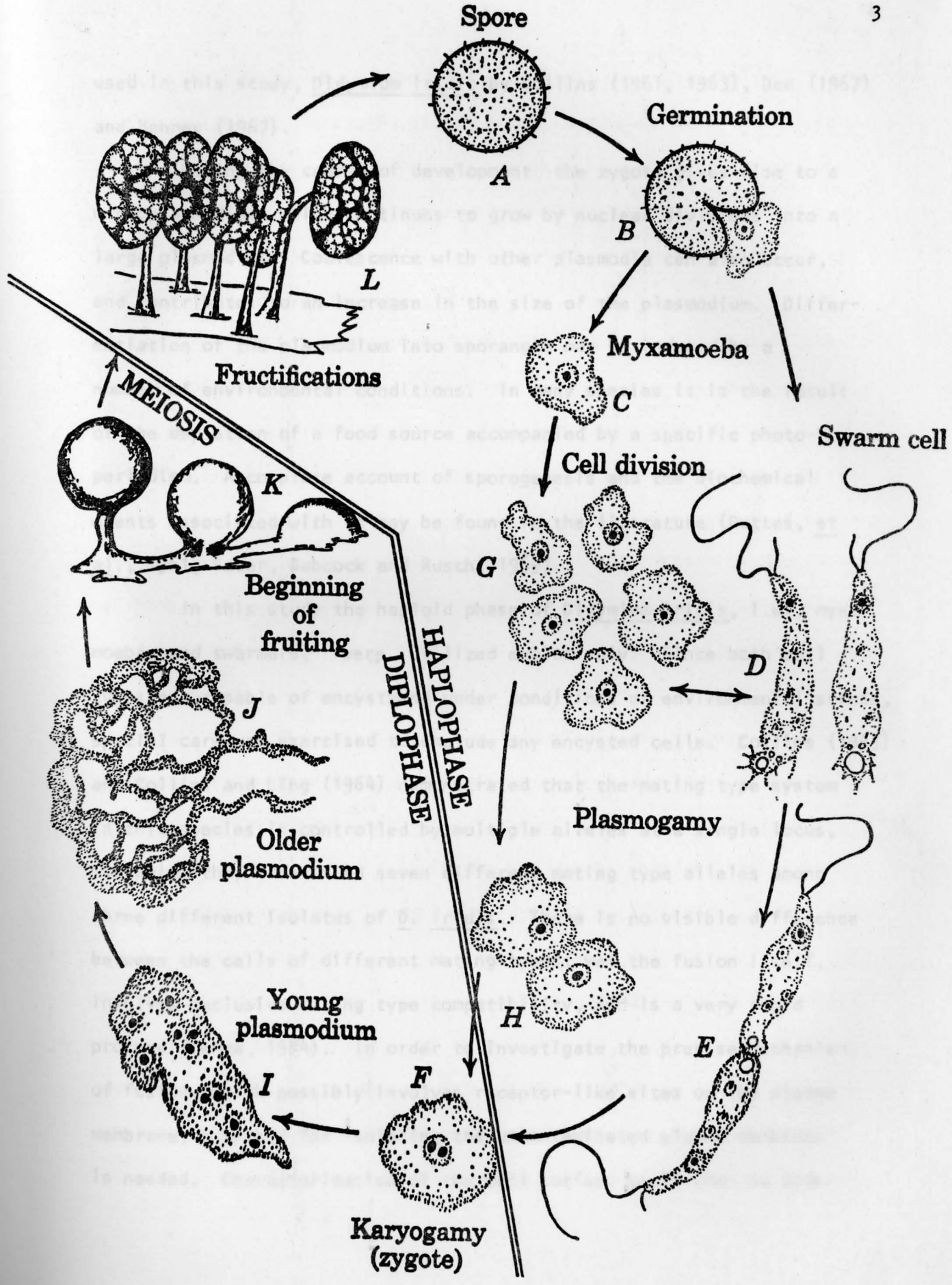


Plate 1.--Life cycle of a Myxomycete, by C.J. Alexopoulos, 1962. Introductory Mycology, by John Wiley and Sons, Inc., New York.


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

used in this study, Didymium iridis by Collins (1961, 1963), Dee (1967) and Henney (1967).

During the course of development the zygote gives rise to a microplasmodium which continues to grow by nuclear divisions into a large plasmodium. Coalescence with other plasmodia can also occur, and contributes to an increase in the size of the plasmodium. Differentiation of the plasmodium 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).

In this study the haploid phase of Didymium iridis, i.e., myxamoebae and swarmers, were utilized exclusively. Since both cell types are capable of encystment under conditions of environmental stress, special care was exercised to exclude any encysted cells. 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, for which they identified seven different mating type alleles among three different isolates of D. iridis. There is no visible difference between the cells of different mating types; yet the fusion itself, involves exclusive mating type compatibility, and is a very rapid process (Yemma, 1974). In order to investigate the precise mechanism of fusion, which possibly involves receptor-like sites on the plasma membrane, a method for isolating the uncontaminated plasma membrane is needed. Characterization of the cell surface could then be under-

taken. This study dealt specifically with the development of a technique for the isolation of the plasma membrane of Didymium iridis through adaptation and modification of physical-chemical separation techniques. The purity of the membranes were then evaluated by chemical and enzymatic assays for substances commonly associated with various cellular structures.

The most widely used separation techniques are based on the density gradient principle. The theory and practice of density gradient centrifugation may be found in reviews by de Duve, Berthet, and Beaufay (1959) and Anderson (1956). Briefly, a sample placed in the centrifuge is fractionated by centrifugal force. The number of different density components present in the sample will determine the number of boundaries which form. A sediment co-efficient can then be calculated for each boundary. The gradient method has proven useful for separation of cell homogenates which are relatively dilute. In fact, the limit of sensitivity of this method exists only as the limit for bioassay of the fractionated component.

Initial attempts in this study utilized the sucrose density gradient method for the isolation of the plasma membranes of Didymium iridis. The literature is most complete for applications of this technique to mammalian cell systems (Emmelot, 1964; Coleman, et al., 1967; Ray, 1970). Korn (1969) provides an extensive review of such methods. Documentation of the success of this method for lower organisms is not quite as extensive. Allen, et al. (1974) and Hoover (1974) have applied the technique to amoebae. Ulsamer, et al. (1971) have used the density gradient method to isolate cell fractions of Acanthamoeba

castellani.

Another technique employed in this study for the partition of cellular components and macromolecules employs a two-phase polymer system, initially developed by Per-Ake Albertsson in 1960. Unlike the density gradient method, in which a particle is separated according to its size and density, Albertsson's technique relies on the surface characteristics of the particle. These systems are prepared by mixing aqueous solutions of two suitably different polymers. Because both phases are aqueous, they are particularly well-suited for the separation of cell particles and bio-molecules. "The phase system should be as mild as possible, meaning consideration must be paid to the water content, ionic composition, osmotic pressure, ability to dissolve out substances from the particles, denaturing effects, etc." (Albertsson, 1960).

The type of two phase system formed depends upon the entropy of mixing for the two polymers. If the interaction between the two types of polymer molecules is repulsive, the system will have its most energetically favorable state when the two polymers go into separate phases. This phenomenon is known as "incompatibility" with the result being that one phase will contain almost one polymer entirely, with the other phase containing the other polymer. Cell particles have a strong tendency to be adsorbed at the interface between the two liquid phases. A sample is usually shaken in the two phases at which time the interfacial tension is very large and can adsorb a great number of particles. As the phases settle, the interfacial area diminishes, but the particles adsorbed tend to remain at the interface. The result

is a concentration of particles between two bulk phases which can be collected easily (Albertsson, 1960). Of the many existing two-phase polymer systems, the dextran-polyethylene glycol system is particularly useful for isolation of larger particulates at the interface. The utility of this method has been demonstrated for the isolation of plasma membranes from L- cells by Brunette and Till (1971), and for rat liver cells by Lesko, et al., (1973). Distinct advantages of this technique over the density gradient method, as demonstrated by this study are the speed and ease of separation. Centrifugations using the two-phase polymers can be done at low speeds and do not require expensive equipment. In addition, the composition of the phases can be manipulated to select for the surface characteristics of the particle to be isolated.

In this study principles of both density gradient centrifugation and the two-phase polymer system were employed in isolation of the plasma membranes of Didymium iridis. The isolated membrane fraction was then assayed for the relative purity of the preparation. The lack of development of an isolation method to date has been due largely to the difficulty of culturing the organism axenically and in large enough numbers. One must eliminate bacterial contamination as well as subcellular particle contamination in any reliable separation technique. The development of such a technique would be invaluable to the continuance of studies of the myxomycetes at the molecular level.

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 from 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 Bacto-Agar in 1 liter of distilled water. The mixture was then 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 5×10^4 to 1×10^6 cells/ml and bacteria numbered from 1×10^9 to 1×10^{10}/ml. The cells were resuspended in 2 to 4 ml of buffer between washings and counts were made using a hemocytometer. Extreme caution was used in observing sterile technique throughout 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 1/2 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 then innoculated with the cells. The culture was placed on a reciprocating shaker bath maintained at 23 degrees C. Cell counts were made every two days and when the density reached approximately 1×10^6 cells/ml, the culture was harvested.

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 50 mM 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 tubes at 70 x gravity for 15 minutes in a swinging bucket 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 relatively 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 bacteria 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 to 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

From this point on, all steps in the procedure were carried out at 0 to 4 degrees C unless otherwise noted. 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 at 1400 revolutions per minute in the swinging bucket rotor of an I.E.C. HN-S centrifuge. The supernatant fraction was designated "S" and the pellet was placed into either the sucrose density gradient or into the two-phase polymer system for further separation.

Isolation of Plasma Membranes

Sucrose Density Gradient Isolation of Plasma Membranes

A discontinuous sucrose density gradient method (Ulsamer, et al., 1971) was modified and initially used as a plasma membrane isolation procedure in this study. The pellet that was previously recovered from centrifugation of the homogenate was re-suspended in 2 ml of 10 percent sucrose and centrifuged at 500 x gravity for 20 minutes in the fixed angle rotor of a Sorvall SS-1 centrifuge. The pellet from this centrifugation was suspended in 10 percent sucrose

once again and centrifuged at 750 x gravity for 20 minutes.

The pellet was then suspended in 1 ml of 25 percent sucrose and layered over 30 percent sucrose. This preparation was spun at 200 x gravity for 20 minutes. After this centrifugation, the 25 percent layer is collected and diluted to 10 percent sucrose with buffer. This is spun at 750 x gravity for 20 minutes. The pellet from this centrifugation is suspended in 50 percent sucrose and layered under 45 percent, 40 percent, and 35 percent sucrose. This was centrifuged at 15,000 x gravity for 12 hours in a refrigerated chamber. Using a Pasteur pipette, plasma membranes were withdrawn from the 40 percent and 45 percent sucrose interface.

Isolation of Plasma Membranes Using a Two-Phase Polymer System

Preparation of the Polymers

The polymers were prepared according to the method of Brunette and Till (1971). The following reagents were used:

200 grams of 20 percent (w/w) Dextran 500 (Pharmacia Upsala) in distilled water

103 grams of 30 percent PEG (polyethylene glycol, Carbowax 6000, Union Carbide) in distilled water

99 ml of distilled water

333 ml of 50 mM phosphate buffer, pH 6.5

80 ml of 0.10M zinc chloride

It was found that the polymers would go into solution more easily if a thick paste was formed by adding only part of the water. The remaining water was added gradually and the polymer solutions

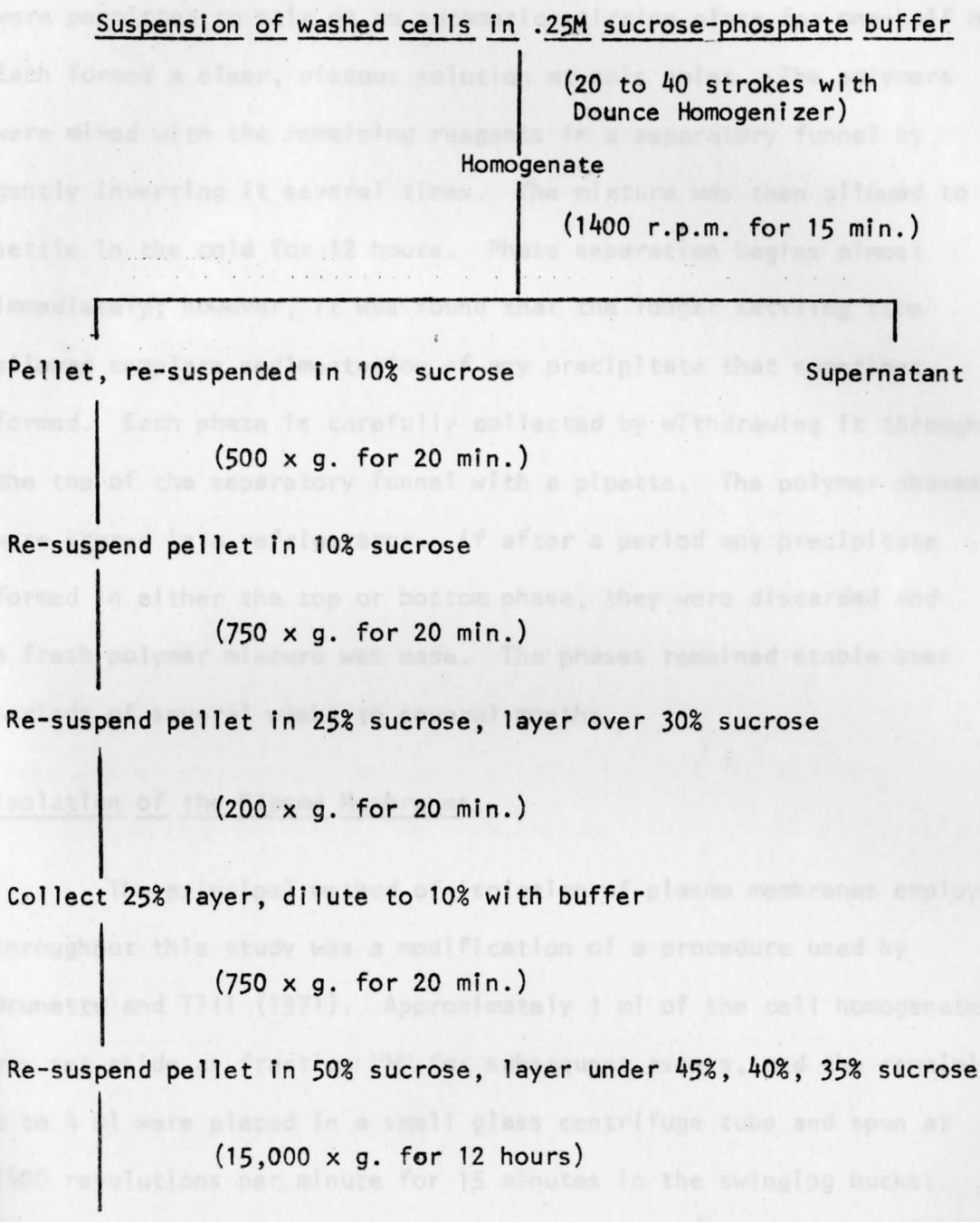


Fig. 1.--Summary of sucrose density gradient isolation of plasma membranes.

were permitted to stir on an automatic stirring plate for one-half hour. Each formed a clear, viscous solution at this point. The polymers were mixed with the remaining reagents in a separatory funnel by gently inverting it several times. The mixture was then allowed to settle in the cold for 12 hours. Phase separation begins almost immediately; however, it was found that the longer settling time allowed complete sedimentation of any precipitate that sometimes formed. Each phase is carefully collected by withdrawing it through the top of the separatory funnel with a pipette. The polymer phases were stored in a refrigerator. If after a period any precipitate formed in either the top or bottom phase, they were discarded and a fresh polymer mixture was made. The phases remained stable over periods of several weeks to several months.

Isolation of the Plasma Membranes

The principal method of isolation of plasma membranes employed throughout this study was a modification of a procedure used by Brunette and Till (1971). Approximately 1 ml of the cell homogenate was set aside as fraction "H" for subsequent assays, and the remaining 3 to 4 ml were placed in a small glass centrifuge tube and spun at 1400 revolutions per minute for 15 minutes in the swinging bucket rotor of an I.E.C. HN-S centrifuge. The supernatant of this centrifugation was designated fraction "S". The pellet was then separated further in the two-phase polymer system.

The pellet was suspended in 2 ml of the top phase of the polymer system. Two ml of the bottom phase were added and the centrifuge tube was inverted several times to allow for maximum interfacial area

between the two phases. The mixture was then spun at 3200 revolutions per minute for 25 minutes. Crude membranes were found at the interface after this centrifugation. The membranes, along with both phases are drawn off with a pipette and re-mixed in another centrifuge tube. The remaining pellet was re-suspended in 3 to 4 ml of water and labelled fraction "P". The crude membranes were spun once again at 3200 revolutions per minute for 25 minutes. This procedure was repeated until a pellet was no longer formed at the bottom of the centrifuge tube. Including the initial centrifugation in the polymers, this was usually accomplished with a total of four centrifugations.

The membranes were then carefully drawn out from the interface with a Pasteur pipette, re-suspended in 3 ml of distilled water, and were spun down at 1000 revolutions per minute for 15 minutes. This step served to wash any adhering polymer free of the membrane. The sedimented membrane pellet was resuspended in 3 to 4 ml of distilled water and designated as fraction "M". All fractions were kept at 4 to 10 degrees C until enzymatic and chemical assays could be performed. Preservation of enzymatic activity of the various cell fractions in this temperature range has been demonstrated in this and other studies (Emmelot, et al., 1964).

Enzymatic and Chemical Assays

One of the major problems encountered in any membrane isolation procedure is that of contamination from other kinds of sub-cellular membranes. A pure preparation of plasma membranes is generally determined by assaying for the absence of the enzyme

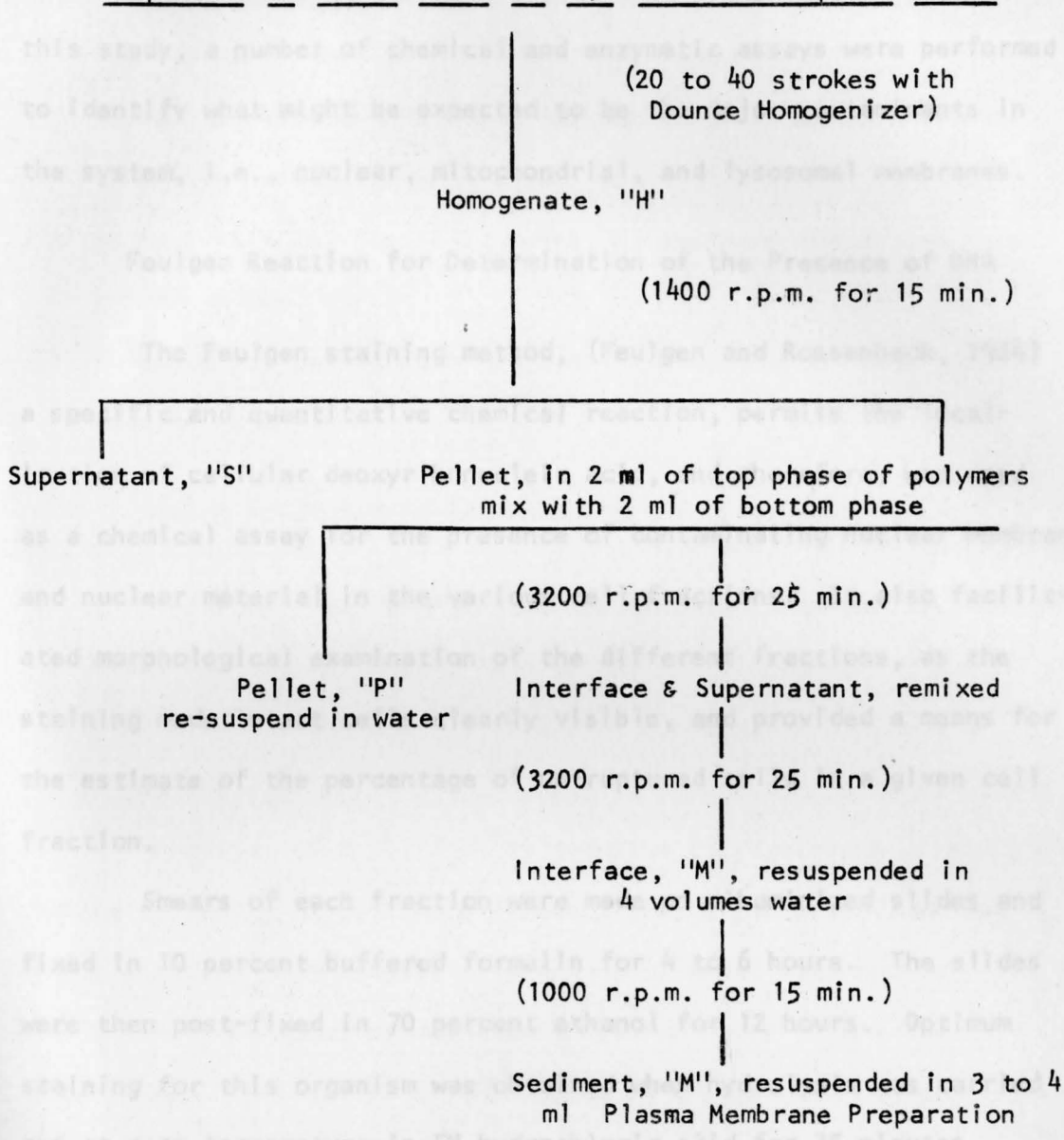


Fig. 2.--Summary of two-phase polymer isolation of membranes.

characteristics associated with the contaminating structures. In this study, a number of chemical and enzymatic assays were performed to identify what might be expected to be the major contaminants in the system, i.e., nuclear, mitochondrial, and lysosomal membranes.

Feulgen Reaction for Determination of the Presence of DNA

The Feulgen staining method, (Feulgen and Rossenbeck, 1924) a specific and quantitative chemical reaction, permits the localization of cellular deoxyribonucleic acid, and therefore, was used as a chemical assay for the presence of contaminating nuclear membranes and nuclear material in the various cell fractions. It also facilitated morphological examination of the different fractions, as the staining made intact cells clearly visible, and provided a means for the estimate of the percentage of un-ruptured cells in a given cell fraction.

Smears of each fraction were made on albuminized slides and fixed in 10 percent buffered formalin for 4 to 6 hours. The slides were then post-fixed in 70 percent ethanol for 12 hours. Optimum staining for this organism was obtained when hydrolysis was carried out at room temperature in 5N hydrochloric acid for 35 minutes (Yemma and Therrien, 1972).

The staining, as modified by Bryant and Howard (1969), was performed as follows:

(1) Slides were hydrolyzed for 35 minutes at room temperature in 5N hydrochloric acid.

(2) Slides were stained in Schiff's reagent (Lillie, 1951) which had been freshly fortified with 10 percent potassium meta-bisulfite

(freshly mixed) for 1 hour. One part potassium meta-bisulfite was used with four parts of Schiff's reagent.

(3) The slides were then brought through three 5 minute rinses of 10 percent potassium meta-bisulfite.

(4) After rinsing in distilled water, slides were dehydrated in a graded ethanol series.

(5) Permanent slides of each fraction were made after they were cleared in xylene.

Spectrophotometric Determination of Cytochrome Oxidase

Of the various enzymes of the electron transport system, cytochrome oxidase was chosen as a marker for mitochondrial contamination. The spectrophtotmetric method of Cooperstein and Lazarow (1950) was utilized.

Thirty ml of a 1.7×10^{-5}M solution of cytochrome c in 0.03M phosphate buffer, pH 7.4, were reduced in an erlenmeyer flask by adding 0.100 ml of a freshly prepared solution of 1.2M sodium dithionite. The solution was shaken vigorously for 2 minutes to remove excess sodium dithionite. Three ml of the reduced cytochrome c solution were pipetted into a quartz cuvette, and 0.04 ml of the tissue homogenate was added. The reactants were mixed by inverting the cuvette, and readings were taken every 30 seconds at 550 nm on a Varian Techtron 635. At the end of three minutes, a few grains of potassium ferricyanide were added to oxidize the cytochrome c completely and the extinction was re-determined. All experiments were run at room temperature.

Assay for the Presence of 5'-nucleotidase on the Plasma Membrane

The presence of 5'-nucleotidase on the plasma membranes of mammalian cells, specifically those of rat liver, has been demonstrated by many investigators (Emmelot, 1964; Coleman, Michell, et al., 1967). In this study, the assay for the presence of 5'-nucleotidase was performed according to the procedure of Michell and Hawthorne (1965).

The substrate, 5'-adenosine monophosphate, was obtained from Sigma Chemical Co. (675-1) and reconstituted with 25 ml of distilled water, to give a 5mM solution. Added to the substrate medium were 2.5×10^{-3} grams of potassium chloride, 2.5×10^{-4} grams of magnesium chloride, and 2.5×10^{-6} grams of sodium potassium tartrate.

Cell fractions of 0.5 ml were incubated for 15 minutes at 37 degrees C in 2 ml of the substrate medium. The reaction was terminated with 1 ml of 25% (w/v) trichloroacetic acid. Inorganic phosphate was assayed in 2 ml of the supernatant by the method of King (1932).

Assay for Inorganic Phosphate

King's test for the presence of inorganic phosphate is a colorimetric one. The following reagents were employed in this test:

5% ammonium molybdate in 15% sulfuric acid

0.2% aminonaptholsulfonic acid (0.5 grams of 1:2:4 acid, 30 g sodium bisulfite, and 6 g sodium sulfite were placed in a flask and brought to 250 ml with distilled water. The solution was then shaken and then filtered. The filtrate should be clear).

Standard phosphate. (Dissolve 2.1935 g of potassium phosphate, monobasic in 500 ml of distilled water. This solution contained 1 mg P_i/ml. A dilute solution is made by diluting 5 ml of the above to 500 ml with water).

To 0.5 ml of each cell fraction, and a phosphate buffer standard, 2 ml of molybdate-sulfuric acid and 0.5 ml of sulfonic acid were added. The volume was brought to 15 ml with distilled water. The contents of the test tube were shaken after each addition. After 5 minutes, absorbance was read at 830 nm on a Varian Techtron 635 spectrophotometer (the absorbance value of the phosphate buffer was subtracted from values obtained for the homogenate and supernatant fractions).

Assay for Acid Phosphatase

Although a small amount of acid phosphatase may be associated with the plasma membrane, it is generally considered to be a characteristic enzyme of lysosomes. The colorimetric test used to identify acid phsophatase in this study is that of Hopkinson, et al., (1964), as modified by Brewer and Sing (1970). Further adaptations were made to accomodate this system and the following procedure was established:

(1) A staining buffer consisting of 0.0075 M phenolphthalein diphosphate in 0.05M citric acid, pH 6.0 was made. (The citric acid buffer was adjusted to pH 6.0 with 2N sodium hydroxide before adding the phenolphthalein diphosphate).

(2) Equal proportions of the staining buffer and 2% Noble agar are mixed and heated to 55 degrees C, or until the mixture

clears. The buffered agar is then poured into plates and allowed to gel.

(3) Samples of 0.1 ml of each fraction along with an acid phosphatase standard, were applied to the surface of the gel and were incubated at 37 degrees C for 3 to 4 hours.

(4) Plates were then exposed to fumes of ammonium hydroxide to give a pink color test.

Protein Measurement with the Folin-Phenol Reagent

Protein determinations using the Folin-Phenol reagent were performed according to the method of Lowry, et al., (1951). Although it has some disadvantages, this method was chosen for its sensitivity over other methods, since small amounts of tissue were used throughout this investigation. The following reagents were used:

Reagent A: 2 percent sodium carbonate in 0.10N sodium hydroxide

Reagent B: 0.5 percent copper sulfate·5 water in 1 percent sodium or potassium tartrate.

Reagent C: Alkaline copper solution. Fifty ml of Reagent A were mixed with 1 ml of Reagent B. This reagent was discarded after one day.

Reagent D: Folin-Ciocalteau phenol reagent (Fisher Scientific Co.) was diluted to 1N.

Standard Protein Solution: 0.04 mg/ml solution of bovine serum albumin in 0.9 percent sodium chloride.

The protein determination was set up in the following manner: (All values in ml)

TABLE I

FOLIN-LOWRY PROTEIN DETERMINATION

	Standard Curve Samples						Cell Fractions			
	1	2	3	4	5	6	H	S	P	M
Reagents										
4.0 mg/ml BSA	.02	.05	.10	.15	.20	-	-	-	-	-
Cell Fractions	-	-	-	-	-	-	.2	.2	.2	.2
0.9% NaCl	.18	.15	.10	.05	-	.20	-	-	-	-
Reagent C	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Reagent D	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

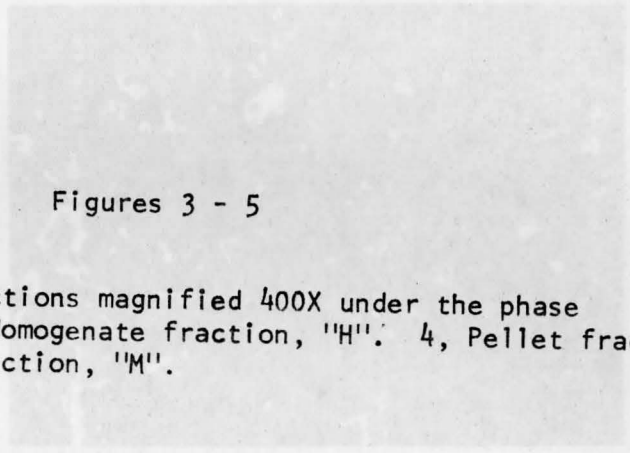
The solutions were mixed in 10 ml test tubes. After Reagent C was added, the tubes were allowed to stand for 10 minutes. Reagent D was added with rapid mixing and the solutions were allowed to stand for 30 minutes or longer. The absorbance of each was read at 750nm on a Varian Techtron spectrophotometer. If absorbance was too great at 750nm, readings were made near 500nm.

RESULTS

Morphological Examination of Membranes

The various steps of the isolation procedure were monitored by light and phase contrast microscopy. It was noted that many of the myxamoebae were somewhat resistant to homogenization and Feulgen staining was employed to insure that the cells did not remain intact during the isolation of the membranes. This procedure permitted the identification of whole cells and bacteria, and in all cases, any structure containing DNA stained an intense violet color. Those cells, whose protoplast had been extracted leaving only the plasma membrane, were not Feulgen positive.

Slides of the homogenate stained by the Feulgen reaction could be seen to contain many cell fragments, occasional intact cells, and a few bacteria which had not been washed free of the membranes. Intense staining of nuclei, nucleoplasm, and other assorted cellular debris was observed. The membrane fragments observed were of various sizes and had a translucent quality in phase-contrast microscopy (see figure 3). The supernatant fraction did not reveal any discernable structures at a magnification of 400X under phase-contrast microscopy. The Schiff's stained slides of this fraction failed to show cellular structures under the light microscope up to magnifications of 1000X. This fraction was later characterized enzymatically.



Figures 3 - 5

Isolated cell fractions magnified 400X under the phase contrast microscope. 3, Homogenate fraction, "H". 4, Pellet fraction, "P". 5, Membrane fraction, "M".

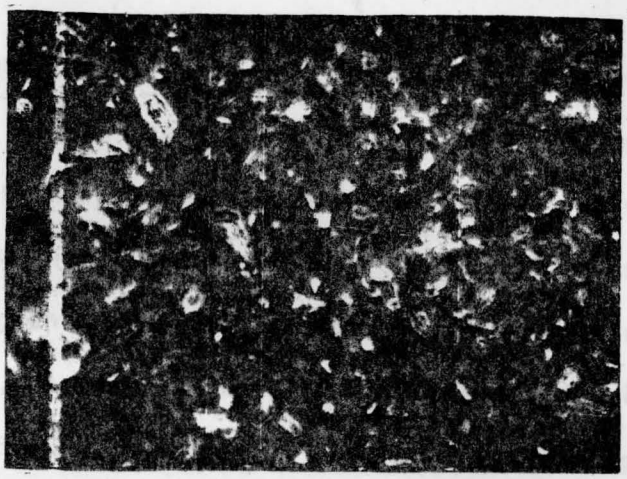


Fig. 3

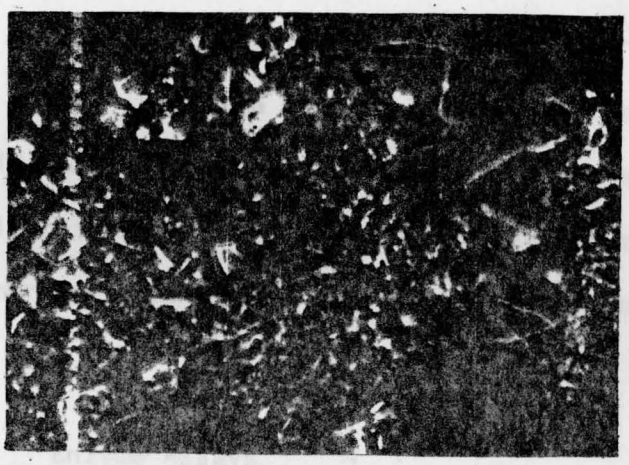


Fig. 4

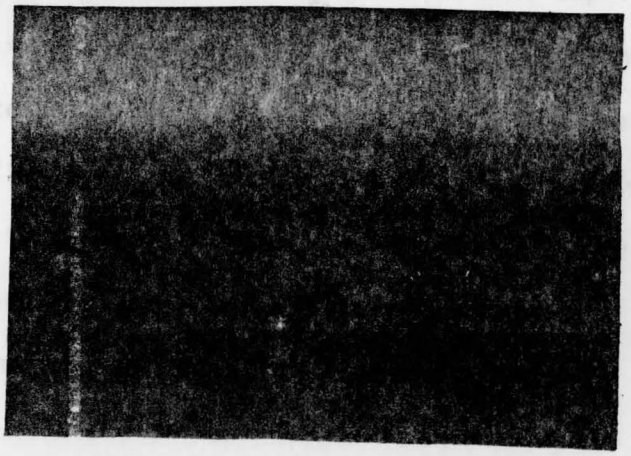


Fig. 5

After the first centrifugation in the two-phase polymer system, a light brownish-white pellet was recovered. The morphological appearance of this sediment under the phase-contrast microscope was very similar to that of the homogenate (see figure 4). Noticeably missing, however, were membrane fragments of an intermediate size. Smaller pieces of fragmented cells, as well as much larger pieces of debris from the corn meal agar medium were present. Once again, staining of a few intact cells and bacteria, and nuclear material was observed.

The membrane preparation appeared at the interface between the two polymers as a fluffy white band. This fraction was devoid of cellular debris, although an occasional bacterium was seen. The plasma membranes which collected at the interface were of a uniform size (see figure 5). Under mild homogenization conditions, there were a greater percentage of whole cell membranes, similar to erythrocyte ghosts, observed. The membrane preparations were consistently noted to be Feulgen negative.

Enzyme Assays

The results of the enzymatic assays are presented as tables. All values represent averages of experiments run in either duplicate or triplicate. Since the data presented in this study are original, and the literature indicates that no plasma membrane isolation technique has ever been established for *D. iridis*, there are no previously established standards for this organism with which to compare the data presented here. Results are presented and will be interpreted relative to one another, and not as absolute values.

Assay for Cytochrome Oxidase

The cytochrome oxidase activities of the various cell fractions were determined in a manner similar to the one Cooperstein and Lazarow (1951) used to assay the cytochrome oxidase content of rat tissue. They found that the oxidation of cytochrome c was a first order reaction, and the enzyme activity could, therefore, be expressed as a decrease in the molar concentration of reduced cytochrome c with respect to time. In this study the data were treated in the following manner: The absorbance (extinction) of the completely oxidized sample was subtracted from the absorbance at any given time. The logarithm of this difference was then plotted against time. Enzyme activity was considered proportional to the slope of the line given by these plots. The results were standardized by dividing the activities by the milligrams of protein present in each sample that was assayed.

The protein content of the samples was determined by the Folin-Lowry method (1951) with a modification for the treatment of data introduced by Stauffer (1975). Unlike the curvilinear relationship which results in the standard curve when absorbance is plotted versus micrograms of protein, he has found that a plot of log absorbance versus log micrograms of protein is linear over a range of 4 to 500 g protein. A linear curve has the advantage of reducing error when reading experimental values from the curve. In this study a linear standard curve was obtained with five serial dilutions of bovine serum albumin by plotting log absorbance versus log micrograms protein (Figure 6). The micrograms of protein present in the

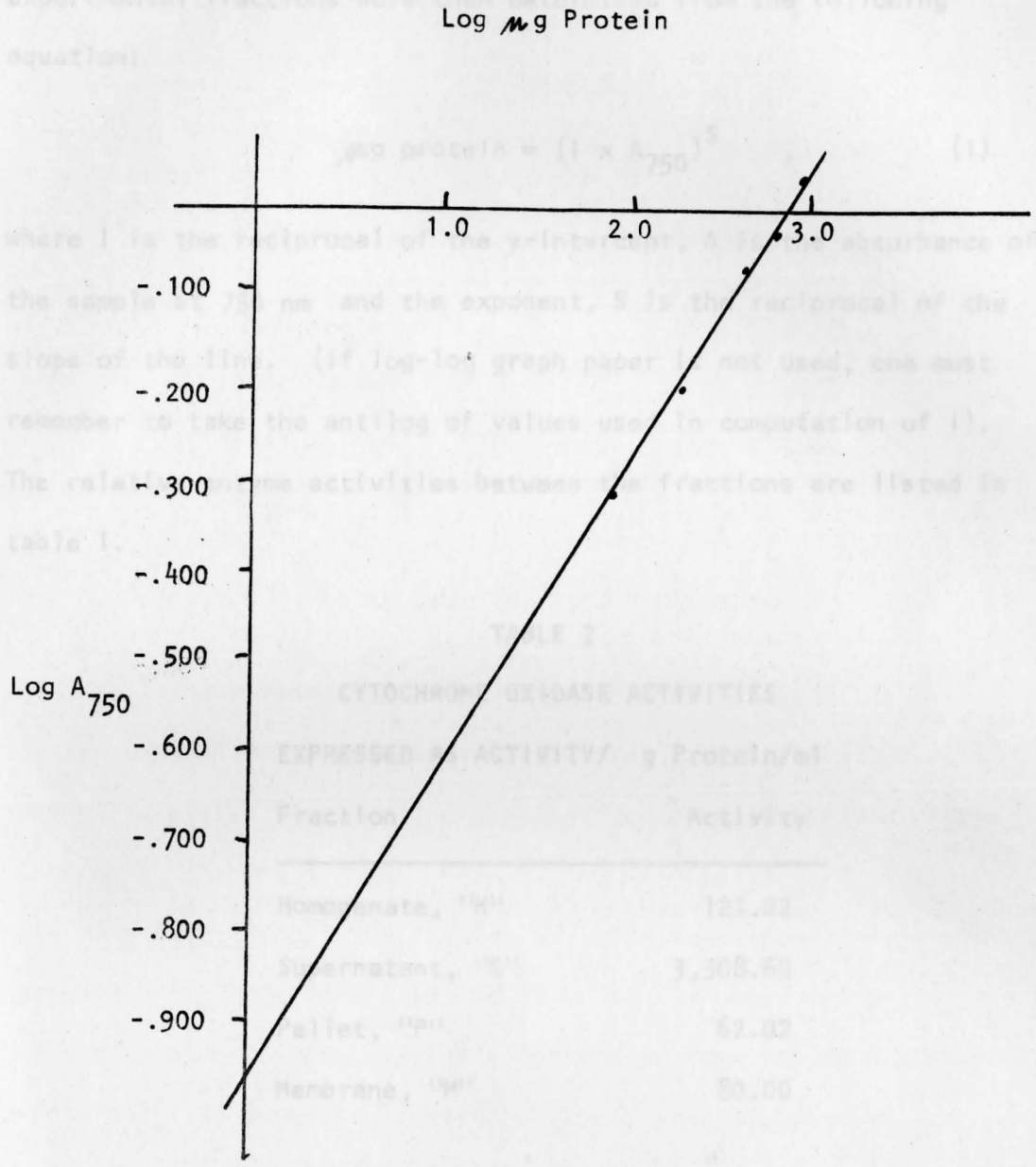


Fig. 6.--Standard curve for the Folin-Lowry determination of protein.

experimental fractions were then determined from the following equation:

$$\mu g \text{ protein} = (I \times A_{750})^{S} \qquad (1)$$

where I is the reciprocal of the y-intercept, A is the absorbance of the sample at 750 nm and the exponent, S is the reciprocal of the slope of the line. (If log-log graph paper is not used, one must remember to take the antilog of values used in computation of I). The relative enzyme activities between the fractions are listed in table 1.

TABLE 2

CYTOCHROME OXIDASE ACTIVITIES

EXPRESSED AS ACTIVITY/ g Protein/ml

Fraction	Activity
Homogenate, "H"	121.02
Supernatant, "S"	3,308.60
Pellet, "P"	62.02
Membrane, "M"	80.00

Assay for 5'-nucleotidase

The activity of 5'-nucleotidase (5'-ribonucleotide phosphohydrolase) was demonstrated indirectly by assaying for inorganic phosphate after the substrate, 5'-adenosine monophosphate was incubated with the various cell fractions. The colorimetric test for inorganic phosphate (King, 1932) yielded the results presented in Table 2. The values are presented as averages of three separate

experiments.

TABLE 3

SUB-CELLULAR DISTRIBUTION OF 5'-NUCLEOTIDASE

Fraction	Absorbance (830nm)
Homogenate, "H"	.063, ± .028
Supernatant, "S"	.006, ± .027
Pellet, "P"	.240, ± .008
Membrane, "M"	.299, ± .007

Color Test for Acid Phosphatase

The presence of acid phosphatase in each fraction was tested for by a qualitative colorimetric reaction. At an acid pH, acid phosphatase will cleave off phosphate from phenolphthalein diphosphate, liberating phenolphthalein which will subsequently turn pink under alkaline conditions. The results of three experimental runs are presented in Table 4. It should be noted that only the pellet fraction gave consistently positive tests for the presence of acid phosphatase, while only the membrane fraction was consistently negative.

TABLE 4

SUMMARY OF ACID PHOSPHATASE ASSAYS

	Fraction				
Run	Std.	H	S	P	M
I	+	+	-	+	-
II	+	-	+	+	-
III	+	-	+	+	-

DISCUSSION

Since a survey of the literature indicated that no successful isolation of the plasma membranes of Didymium iridis had been accomplished previously, it was the original intent of this study to isolate the plasma membranes of D. iridis using a classical biochemical approach to cellular fractionation. Of the many proven techniques, it was thought that the density gradient centrifugation method might best be modified and applied to this particular cell system. Most sucrose density gradient techniques rely on high speed or ultra centrifuges operating at low temperatures. The instrument used must be capable of generating enough revolutions per minute to permit cellular components with small density differences to be separated over varying intervals of time. In many cases it is possible to use lower speed centrifugations to effect a separation by increasing the spinning time proportionately. In biological systems, however, extremely high speeds over long intervals of time, pose a problem, for these conditions may lead to deterioration and loss of the enzymatic activity of the various cellular components essential to their identification. In addition, the temperature requirement of 0 to 4 degrees C is difficult to maintain during the various steps of the isolation procedure.

The initial plasma membrane isolations were done employing a Sorvall SS-1 type centrifuge, as previously described in detail in the materials and methods section. It was found that extreme speeds

(greater than 31,000 x gravity) and relatively short centrifugation times, resulted in a low yield of the desired fraction, and did not entirely remove any bacterial contamination that might be present, thus making the necessary assays of marker enzymes exceedingly difficult. At lower speeds, centrifugation times became so long as to be impractical and resulted in considerable damage to cell fractions. The low yields may have been due, in part, to the fact that a fixed angle head rotor was used instead of a swinging bucket type rotor. In his review of gradient centrifugation, Moore (1969) states that "horizontal tubes are usually preferable to angle heads because there is less convection and less damage to delicate particles from sliding along the tube wall." It is possible that the yield was somewhat decreased by such convection effects which caused a greater number of membranes to sediment along with the cellular debris rather than at the 40/45 percent sucrose interface. The technique was abandoned since it could not be modified to yield the amount of membranes, nor could it remove all bacterial contamination that were necessary for an accurate biochemical characterization.

A separation method that could utilize low-speed centrifugation equipment and yield sufficient quantities of undamaged plasma membranes free from bacterial contaminants was needed for reliable enzymatic and chemical analysis. Brunette and Till (1971) successfully applied the aqueous two-phase polymer systems for partition of cell particles developed by Albertsson in the early 1960's to the isolation of plasma membranes of "L" cells. Their method was modified for use in this study by lowering centrifugation speeds to a third of the speeds prescribed by Brunette and Till. When the centrifugations times were

increased slightly, the lower speeds worked quite effectively. This modification was probably successful because much smaller volumes of tissue and polymers were being used than were used by Brunette and Till. Consequently, the mixture of polymers formed two phases more rapidly and under less centrifugal force. The lower centrifugation speeds made it much easier to maintain cell preparations between 0 and 4 degrees C. It was also found that the phosphate buffer system used in the culturing of D. iridis could be substituted for the one used by Brunette and Till, with no effect on the isolation itself. It was not known to what degree ions might interact and what effect this interaction might have upon the membranes if the buffer system of the polymers was different from that of the homogenate. The particular concentration of sucrose-phosphate buffer used prevented osmotic shock to the cells and resultant membrane damage, especially in the early stages of homogenization and isolation. This permitted the recovery of relatively intact membranes. The two-phase polymer systems embrace many practical aspects of an isolation problem in that they provide a rapid, effective means for recovery of cell fractions using only low speed centrifugations.

There are many kinds of polymer systems, all of which behave according to the general principles of phase separation. In theory, if two polymers are mixed in water, phase separations will occur above a certain concentration of each, while at other concentrations, a homogeneous mixture will result. There is a point, K, called the critical composition, at which the compositions and volumes of the phases are theoretically equal. Even a very small change in total composition from below to above point K results in a change from a

one-phase to a two-phase system, with nearly identical volumes of the two phases found (Albertsson, 1960).

The choice of a dextran-polyethylene glycol system (Brunette and Till, 1971; Lesko, et al., 1973) is a good one in that it can be used at concentrations which are far removed from its critical composition. This permits a great deal of flexibility because the system becomes less sensitive to changes in polymer composition, temperature, etc. (Albertsson, 1960). In addition, Albertsson has found that the further a system is from its critical point, the greater its interfacial tension. This is of particular importance, because larger particles, such as the plasma membranes, tend to be adsorbed at the interface of two-phase polymer systems.

With the dextran-polyethylene glycol system a high yield of membranes was recovered from the interface between the polymers, when 2 ml of each phase were used. These volumes were found by trial and error. Apparently, the polymer system can be "over-loaded" with the sample to be separated and gravity then causes clumps of membranes to sediment out of the interface (Albertsson, 1960). Results of chemical and enzymatic assays indicate that the plasma membranes which were recovered using this method were relatively free of contaminants.

Perhaps the best proof of the purity of the membranes from nuclear contaminants came from microscopic examination of the Feulgen stained slides made of each fraction. While the homogenate and pellet fractions exhibited intense staining, the membrane fractions were consistently Feulgen negative. The degree of fragmentation of membranes depended on the amount of homogenization and the type of homogenizer used. In any case, membrane fragments found at the interface

were always of a uniform size, which would support the theory that two-phase polymer separations of cell particles are a surface phenomenon. Fragments of varied sizes could be seen in the homogenate and pellet fractions. The intense staining of both of these fractions is probably due to the staining of bacteria, nuclei and nucleoplasm present in them.

The results of the cytochrome oxidase assays suggest that the mitochondria are concentrated in the supernatant fraction, as it consistently showed much higher activity than any of the other fractions. The homogenate might also be expected to display a great deal of activity; and, although it was the fraction with the second highest amount of cytochrome oxidase activity, it was much less than that of the "S" fraction. This may be an inherent characteristic of this method of isolation. Only a small fraction of the homogenate is set aside for assays while the greatest part is separated into the "S" fraction and the pellet, which goes into the polymer system for further separation. The mitochondria remain behind, concentrated in the supernatant fraction, while membranes and other sub-cellular components and debris form the pellet. All of these components which are initially present in the homogenate would reduce activity of a specific enzyme such as cytochrome oxidase. The comparatively small amount of cytochrome oxidase activity recorded for the pellet and membrane fractions indicates that mitochondrial contamination of the membranes is not a serious problem.

Although there was great disparity in the values obtained for the cytochrome oxidase activity for each fraction between experimental runs, the same relative activities between fractions were observed

during each run; i.e., the supernatant fraction always showed the greatest activity, followed by the homogenate. Membrane and pellet fractions always exhibited minimal activity. The disparity of values for each fraction between runs is probably a result of the initial number of cells homogenized, since activity was determined as a function of the protein present in each fraction assayed. Whether homogenate is a result of a large or small harvest of cells would change the magnitude of the activity drastically, as the same volumes were used for every assay. Cooperstein and Lazarow (1951) suggested that differences in values between the experimental runs may also be due to the manner and degree of homogenization.

The results of the 5'-nucleotidase assay are somewhat more difficult to interpret. This particular phosphohydrolase is considered a characteristic marker for the plasma membrane of mammalian cells as well as many non-mammalian type cells. As previously stated, the isolation of the plasma membrane of D. iridis has never been reported in the literature; therefore, it was not known if the enzyme was present, and could be used as a reliable marker for the plasma membrane in this organism. The actual assay for 5'-nucleotidase was employed as an indirect colorimetric test for the presence of inorganic phosphate. Because a phosphate buffer was used for homogenization, the homogenate and supernatant fractions must have the absorbance of the plain buffer subtracted from their absorbance values. When this is done, their absorbances are very close to zero, suggesting the absence of the enzyme in these fractions. As indicated in the results, the purified plasma membrane fraction exhibited an extremely high absorbance value in D. iridis. It appears that the enzyme is an

important constituent of the plasma membrane of this organism and can be used as a reliable marker for it.

The pellet fraction also exhibited a high absorbance value for the marker enzyme, although not as high as the plasma membrane fraction. This is easily explained as this fraction contains many variable sized pieces of membrane, and it is not unlikely that it would demonstrate 5'-nucleotidase activity. There is a possible source of error here that must be guarded against when employing this assay. It is possible that adsorbed polyethylene glycol on the membrane surface may give an elevated phosphate determination (Leskó, et al., 1973). It is important to wash the membranes thoroughly with distilled water after the final isolation.

Finally, the results of the assay for acid phosphatase suggest that there is no gross contamination of the membrane fraction by lysosomes. The qualitative test for the presence of acid phosphatase was consistently negative for the membrane fraction and positive for the pellet fraction. The supernatant and homogenate fraction showed occasional activity. As the release of lysosomal enzymes accompanies disruption and deterioration of the cell, its presence in the H, S, and P fractions can be expected.

This study has served to demonstrate that a modified dextran-polyethylene glycol two-phase polymer system provides a good yield of membranes which are relatively free from other sub-cellular contaminants, and is preferable to the sucrose density gradient method. It is a highly practical method for the cell system used in this study for a number of reasons. Since separation is accomplished by virtue

of surface characteristics rather than by density, bacterial contamination in the final membrane preparation was much rarer than was observed using the sucrose density gradient method. This is an especially important advantage of this technique, since an axenic medium has not yet been defined for the culture of Didymium iridis. In addition, the entire isolation procedure can be completed in just over 2 hours. This prevents excessive deterioration of cell fractions and subsequent loss of enzymatic activity. Albertsson (1960) has found that the polymers actually exert a protective effect over the cell particulates. Finally the aqueous two-phase polymer system can be used to effect separations using only inexpensive, low-speed centrifugation equipment.

The flexibility of this method has practically unlimited potential for use in various biological systems, and, investigation of the method itself is far from being exhausted. In its application to the system under investigation in this study, it has provided a rapid and easy means for the isolation of the plasma membranes of Didymium iridis. This was previously considered a tedious, and nearly impossible task. This accomplishment is significant because it paves the way for a little explored area in myxomycete research, namely, the biochemical characterization of the cell surface. This type of knowledge might ultimately explain some of the fascinating behavior displayed by this truly unique organism, which in many cases must involve the plasma membrane.

BIBLIOGRAPHY

Albertsson, Per-Ake. 1960. Partition of Cell Particles and Macromolecules. John Wiley and Sons, Inc., New York.

Albertsson, Per-Ake. 1970. Partition of cell particles and macromolecules in polymer two-phase systems. Advances in Protein Chemistry 24:309-341.

Alexopoulos, C.J. 1962. Introductory Mycology. Second edition. John Wiley and Sons, Inc., New York. 613 p.

Allen, H.T., C. Ault, R. Winzler, J.F. Danielli. 1974. Chemical characterization of the isolated cell surface of Amoeba. The Journal of Cell Biology 60:27-38.

Anderson, N.G. 1956. Physical Techniques in Biological Research. In: G. Oster and A.W. Pollister (eds.) Volume III. Academic Press, New York.

Brewer, G.J. and C.F. Sing. 1970. An Introduction to Isozyme Technique. Academic Press, New York. 127 p.

Brunette, D.M. and J.E. Till. 1971. A rapid method for the isolation of L-surface membranes using an aqueous two-phase polymer system. Journal of Membrane Biology 5:215-224.

Bryant, R., and K. Howard. 1969. Meiosis in the oomycetes: I. A microspectrophotometric analysis of nuclear deoxyribonucleic acid in Saprolegnia terrestris. American Journal of Botany 56:1075-1083.

Coleman, R., R.H. Michell, J.B. Finean, and J.N. Hawthorne. 1967. A purified plasma membrane fraction isolated from rat liver under isotonic conditions. Biochimica et Biophysica Acta 135:573-579.

Collins, O.R. 1963. Multiple alleles at the incompatibility locus in the myxomycete Didymium iridis. American Journal of Botany 50:477-480.

Collins, O.R. 1961. Heterothallism and homothallism in two myxomycetes. American Journal of Botany 48:674-683.

Collins, O.R. and H. Ling. 1964. Further studies in multiple allelomorph heterothallism in the myxomycete Didymium iridis. American Journal of Botany 51:314-317.

Cooperstein, S.J. and A. Lazarow. 1951. A microspectrophotometric method for the determination of cytochrome oxidase. Journal of Biological Chemistry 189:665-670.

de Duve, D., J. Berthet and H. Beaufay. 1959. Progress in Biophysics. J.A.V. Butler and B. Katz (eds.) Vol. IX. Pergamon Press, London.

Dee, J. 1960. Genetic analysis of a myxomycete. Ph.D. Dissertation. The University of Glasgow, Glasgow, Scotland.

Emmelot, P., C.J. Bos, E.L. Benedetti, and P.H. Rumke. 1964. Studies on plasma membranes: I. Chemical composition and enzyme content of plasma membranes isolated from rat liver. Biochimica et Biophysica Acta 90:126-145.

Feulgen, R., and H. Rossenbeck. 1924. Mikroskopisch-Chemischer Nachweiss einer Nuclein sauve von Typus der thymonucleinsauve und die darauf berhende elektive Farburg von Zellkernen in mickroskopischen Praparaten. Z. Physiological Chemistry 135:203-248.

Gray, W., and C.J. Alexopoulos. 1968. Biology of the Myxomycetes. The Ronald Press Co. New York. 288 p.

Guttes, E., S. Guttes, and H. Rusch. 1961. Morphological observations on growth and differentiation of Physarum polycephalum grown in pure culture. Developmental Biology 3:588-614.

Henney, M.R. 1967. The mating type system of the myxomycete Physarum flavicomum. Mycologia 59:637-652.

Hoover, R.L. 1974. Surface characterization of two amoebae relative to cell adhesion. Experimental Cell Research 87:265-276.

Hopkinson, D.A., N. Spencer, and H. Harris. 1964. Genetical studies on human red cell acid phosphatase. American Journal of Human Genetics 16:141-154.

King, E.J. 1932. The colorimetric determination of phosphorus. Biochemical Journal 26:292-297.

Korn, Edward D. 1969. Cell membranes: structure and synthesis. Annual Review of Biochemistry 38:263-288.

Lesko, L., M. Donlon, G.V. Marinetti and J.D. Hare. 1973. A rapid method for the isolation of rat liver plasma membranes using an aqueous two-phase polymer system. Biochimica et Biophysica Acta 311:173-179.

Lillie, R.D. 1951. Simplification of the manufacture of Schiff reagent for use in histochemical procedures. Stain Technology 26:163.

Lowry, O.H., N.H. Rosebrough, A. Farr, and R.J. Rose. 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193:265.

Michell, R.H., and J.N. Hawthorne. 1965. The site of diphosphoinositide synthesis in rat liver. Biochemical and Biophysical Research Communications 21(4):333-338.

Moore, D.H. 1969. Part B. Physical chemical techniques. In: Physical Techniques in Biological Research. Vol. II. pp/ 285-315. Academic Press, New York.

Pickels, E.G. 1943. Sedimentation in the angle centrifuge. Journal of General Physiology 26:341-360.

Sauer, H.W., K.L. Babcock, and H.P. Rusch. 1969. Sporulation in Physarum polycephalum. Experimental Cell Research 57:319-327.

Stauffer, C.E. 1975. A linear standard curve for the Folin-Lowry determination of protein. Analytical Biochemistry 69:616-648.

Ray, T.K. 1970. A modified method for the isolation of plasma membrane from rat liver. Biochimica et Biophysica Acta 196:1-9.

Ulsamer, A., P.L. Wright, M.G. Wetzel, and E.D. Korn. 1971. Plasma and phagosome membranes in Acanthamoebae castellani. The Journal of Cell Biology 51:193-215.

Yemma, J.J., and C.D. Therrien. 1972. Quantitative microspectrophotometry of nuclear DNA in selfing strains of the myxomycete Didymium iridis. American Journal of Botany 59:828-835.

Yemma, J.J., C.D. Therrien, and S. Ventura. 1974. Cytoplasmic inheritance of the selfing factor in the myxomycete Didymium iridis. Heredity 32:231-239.

Yemma, J.J., and C.D. Therrien. 1974. Quantitative cytochemical analysis of nuclear DNA in zygote nuclei of the myxomycete Didymium iridis. American Journal of Botany 61:26.