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ABSTRACT

Inducible Cell Fusion Receptor Analysis
of Isolated Plasma Membrane Fractions
of the Myxomycete **Didymium iridis.**

Joseph Michael Restivo
Master of Science
Youngstown State University, 1992

Data are presented which focus on changes that occur in the cell surface glycoproteins of the myxomycete **Didymium iridis**, which permit cell fusion to occur in previously induced cells. It was therefore necessary to develop a reliable method for the isolation of pure plasma membrane fractions, prior to the characterization of mating and fusion factors. Methods of chromatographic analysis of isolated plasma membrane fractions of pre-fusion (uninduced) versus fusion competent (induced) myxamoeba were carried out. Previous studies have shown that fusion between competent myxamoeba of compatible mating types can be blocked by the lectin concanavalin A. Concanavalin A preferentially binds glycoproteins thus indicating the biochemical character of this fusion receptor (Yemma and Soltis, 1988). Yemma and Perry (1985) provided evidence indicating that compatible cells of **Didymium iridis** must become competent to fuse either by attaining a critical cell density or by being induced into competency by an already fusion-competent population. Induction is initiated by a diffusible organic molecule produced by cells of the same mating type (self-induction)

having attained a critical density of 1 x 10 E+05 cells/milliliter. Cells most suceptible to induction are in the G_1 phase of interphase in the cell cycle (Yemma and Stroh, 1991).

Isolation of the plasma membrane of cells was carried out by modification of a technique by Barden et al. (1983), utilizing density gradient centrifugation in a linear sucrose gradient. This technique, which is a hybrid of isopycnic and rate zonal centrifugations, produced a highly pure membrane fraction. Location of the isolated plasma membrane fraction(s) was done by enzymatic assay of the marker enzyme 5'-nucleotidase. The major plasma membrane fraction was found to be located in the 40% (w/v) sucrose gradient at a mean sucrose density of 44.5% (w/v). Purity of membrane fractions was determined by screening for the presence (or absence) of marker enzymes for membranes other than the plasma membrane.

It was initially hypothesized that a specific membrane fusion receptor would be present on induced cells or cells made competent to fuse, and therefore absent on uninduced cells. HPLC analysis of the membrane glycoproteins of these two respective isolated plasma membrane fractions should produce spectra upon analysis, indicative of this phenomenon. Results of HPLC analysis did indeed demonstrate differences in the plasma membrane glycoprotein profiles of induced versus uninduced cells. Evidence presented indicates that during the induction period prior to fusion (Yemma and Perry, 1985), the plasma membrane undergoes dynamic changes in which membrane

fusion factors are made active through conformational changes of specific membrane proteins which occur on the membrane surface. The conformational changes that occur would account for these differences in HPLC analysis of membrane glycoproteins in induced versus uninduced plasma membrane fractions. Future studies will seek to characterize membrane glycoprotein changes which allow cells to become competent to fuse.

This thesis is dedicated to all those persons who believed in me and were willing to give me the opportunity that others would not. To them I owe a great debt of gratitude for their constant support, encouragement, and also their ability to tolerate and motivate me during the many frustrating times that completing this master's program provided. So especially to my Mom and Dad, my brother Todd, my grandmother, my girlfriend Tricia, and the rest of my family, thank you all for being there for me the most; without each of you, accomplishment of this goal would never have been possible. This thesis is also dedicated to my three grandparents who never had the opportunity to see me come this far. I hope that the person I've become and all that I've accomplished thus far in my life would have made each of you very proud.

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Many individuals were involved in assisting me in the completion of this project. In gratitude, I would like to acknowledge each of them in thanks for their help and I apologize to anyone whose name has been unintentionally omitted. First and foremost, thanks to Dr. John J. Yemma for providing me with the opportunity to prove my ability and regain my confidence. Without his guidance and ability to motivate me whenever I needed it, completion of this project would not have been possible. Next, to Dr. Anthony Sobota, Dr. James Toepfer, and Dr. Paul Van Zandt for taking the time to serve on my thesis defense committee. I also want to thank Dr. Sobota for use of his ultracentrifuge. Additional thanks go to Paul, Dan, Mike, and Laura in the Microbiology lab for providing the media necessary for culturing of the organisms for this project. Thanks also to Scott Howell and Mike Berk as well as all of the other graduate students in Dr. Yemma's lab for a good time and a great deal of help over these past three years, without which this experience would have been much less enjoyable. Finally, thanks to the secretaries in the biology, allied health, and CAST offices; and all undergraduate students who worked in our lab and assisted in the completion of this project.

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CHAPTER I

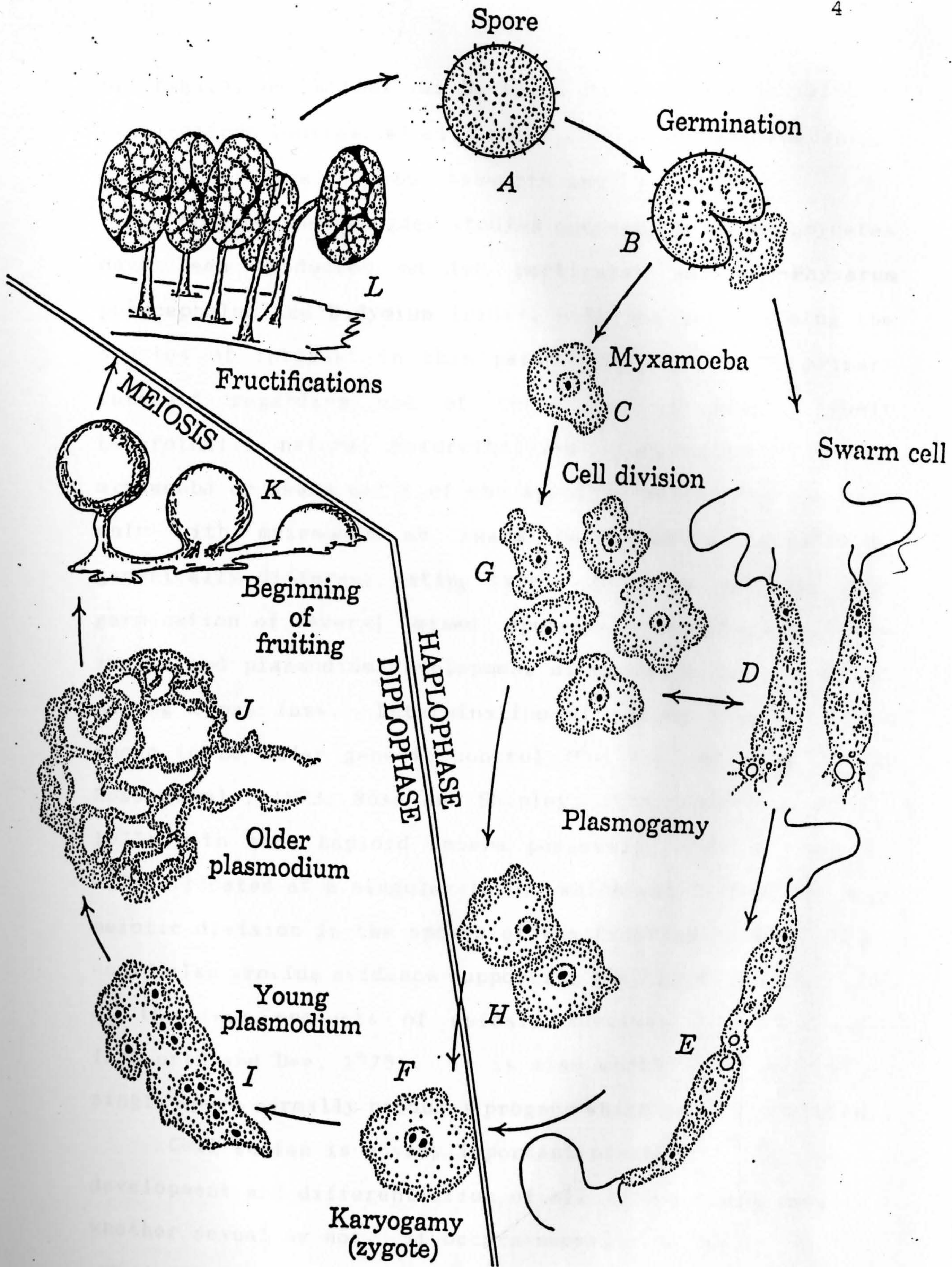
Introduction

The scientific literature indicates that an ongoing search has been carried on in laboratories throughout the world, in the hope that some type of nearly ideal investigative system including an ideal organism might be found to accommodate an array of studies involving developmental biology. In retrospect, nearly two centuries ago, a Swedish botanist named Elias Fries unknowingly stumbled upon an organism of this type which he observed as "a slimy mass on moist leaves" (Alexopoulos and Koevenig, 1962). Later, examination of this slimy mass revealed its transformation into an abundance of small cylindrical fruiting bodies. Thus, two stages in the life cycle of this organism had been observed--plasmodium and fruiting bodies. Some fifty years later, the renowned German mycologist Anton De Bary (early 19TE century) observed the remaining stages in the life cycle of this organism after germinating spores from this "true slime mold". **Didymium iridis**, an eukaryotic organism, is neither a true plant, nor is it in all respects an animal, but instead it belongs to a classification of organisms known as the Mycetozoans which possess both fungal and protozoan characteristics in their life cycles. Slime molds are so called "primitive" eukaryotes which exhibit a basic life cycle in what is, perhaps, its simplest form. This life cycle is characterized by a uninucleate amoeboid stage which can

develop into a multinucleate or multicellular stage which can in turn give rise to a fruiting body containing dormant spores (Ashworth and Dee, 1975). The amoeboid stage illustrates the protozoan animal-like characteristics of the life cycle while the fruiting body stage resembles those characteristics of the lower fungi.

The major consideration as to why this organism represents an ideal system for studies involving developmental stages on the cellular level, is due to its unique life cycle. Both haploid and diploid stages occur in its life cycle which are accompanied with profound developmental changes over a relatively short time. Development of both gametic, zygote and vegetative stages as well as other dramatic developmental stages can be witnessed over a very short time period. The haplophase of the life cycle begins with the release of uninucleate, desiccant-resistant spores from mature fruiting bodies induced by a force of nature such as wind or rain. Under conditions of sufficient moisture, these spores will germinate and give rise to a uninucleate amoeboid form of this respective organism. Two interconvertible protozoan forms can exist in this stage depending on the environmental conditions present. Motile biflagellate swarm cells are predominant under aqueous conditions, whereas drier conditions (such as those found on nutrient agar) favor the irregularly shaped rounded-up form of the myxamoeba. Whether cultured in liquid or on nutrient agar, these amoeboid forms feed on bacteria and mitotically divide until a log phase population is reached.

Figure 1. Life Cycle of Didymium iridis, by C. J. Alexopoulos, 1962. Introductory Mycology, by John Wiley and Sons. Inc., New York p. 72.


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

biochemical mechanisms can be found in several scientific publications (Guttes, et al., 1961; Alexopoulos and Koevenig, 1962; Sauer et al., 1969; Ashworth and Dee, 1975).

Most of the recorded studies concerning the myxomycetes have been conducted on two particular species--**Physarum polycephalum** and **Didymium iridis**, with the latter being the species of interest in this particular study. Of primary interest regarding use of these two strains is their heterothallic nature. Heterothallism refers to the ability of myxamoeba or swarm cells of one specific mating type to fuse only with myxamoeba or swarm cells of a compatible, genetically different mating type. In these species, only germination of several "mixed" spores can result in possible zygote and plasmodium development as opposite or compatible mating types fuse. Determination of mating type has been shown to be under genetic control (Collins and Ling, 1972; Ross et al., 1973; Ross and Shipley, 1973; Adler and Holt, 1975) with each haploid amoeba possessing only a singular allele located at a singular locus, which was derived through meiotic division in the spores of the fruiting bodies. This would also provide evidence supporting the claim that only one of the four products of meiosis survives in each spore (Ashworth and Dee, 1975). It is also worthy of note that a single spore normally produces progeny which are self fertile.

Cell fusion is a very important process in the development and differentiation of all living organisms, whether sexual or not. It occurs normally in the

polykaryocytes of bone and muscle as well as in zygote formation of all diploid organisms including those organisms exhibiting an atypical method of genetic recombination, as may occur in some protozoa and fungi. In **D. iridis**, plasma membrane fusion (and subsequent zygote and plasmodium formation) is controlled by a multiple allelic system which requires the presence of two different alleles at the mating locus. Genetically, **D. iridis** exhibits a bipolar multiallelic mating system (Yemma and Stroh, 1991). Collins and Ling (1968) postulated that the mating locus functions as a regulator gene that is either depressed or corepressed depending on the presence or absence of an unidentified inducer substance. The inducer substance has subsequently been isolated and its physiology described by Yemma and Stroh (1991). It is produced during normal development by amoeba of compatible mating types to either trigger differentiation into the plasmodial stage by its presence or block plasmodial formation through repression of specific genes in its absence. Prior to this proposed mechanism, the majority of research on gametic and somatic fusion had been done using highly differentiated gametes with great morphological differences (Colwin and Colwin, 1967), in which complexities of morphology may have obscured the basic membrane fusion patterns (Ross et al., 1973), or using somatic cells from complex mammalian cell cultures. Fusion between gametes is usually characterized by the ability of the gametes to recognize and fuse only with genetically compatible

partners, while somatic cell fusion usually involves removal of a barrier that serves as a protective mechanism to prevent fusion between non-compatible mating types. Utilizing data collected from studies on virus-induced polykaryocytes in mammalian cell cultures, Poste and Allison (1971), hypothesized that membrane fusion in any biologic system occurs by a common mechanism. Their membrane fusion reaction theory emphasized the immense biological significance of this process at both the cellular and subcellular levels. If control of all cellular fusion (sexual and somatic) could be attributed to a common mechanism or through a common mating locus, then **D. iridis** could serve as an excellent model for its possible elucidation. Studies have been previously conducted on plasmodial mycetozoans concerning the multiple allelic basis of sexual and somatic incompatibility (Collins, 1963; Dee, 1966; Ling,1971; Collins and Haskins, 1970), but minimal research has focused on the physiological and biochemical mechanisms associated with the induction of membrane fusion receptors. A model system is necessary postulating the induction of fusion as the result of cell surface changes initiated by chemical communication through inducer molecules which thus would permit both the recognition and discrimination of compatible mating types, as well as the elucidation of an actual membrane fusion mechanism (Ross and Cummings, 1970). Absence of an assay to quantify the rate of fusion in a cell population accompanied by the fact that both gametic and somatic fusions are subjected to multi-allelic

genetic control had delayed formulation of an adequate hypothesis that would encompass all possibilities. In **D. iridis**, sexual fusion is a function of the recognition of a genetically different compatible mating type while somatic fusion is a function of the recognition of both a phenotypically and genotypically indistinguishable self (Ross et al., 1973).

A further complication regarding the mechanics of fusion can be attributed to the interconvertible nature of the haploid myxamoeba and swarm cells. Yemma and Perry (1985) provided evidence that fusion between myxamoeba, and also between swarm cells, did not exhibit the same mechanistic pattern. This information was very crucial since any slight variance in the environmental conditions could induce these haploid gametes to interconvert and thus be very disruptive to investigations requiring that all cells be in the same state, thus limiting the number of variable factors. Ross et al. (1973) also provided a detailed explanation of the importance of cell culturing by providing evidence indicating that neither the rate nor the amount of fusion was affected by the presence of bacteria as a food source on solid agar media. In contrast, they also indicated that controlling the concentration of amoeba that are initially plated out on nutrient agar media is vital because variability of any type affected the timing of fusion at subsequent matings, suggesting that the number of cell generations during culturing may affect the maturity and subsequent fusion

readiness of the amoeba. Further evidence was provided by results that indicated a relationship between the cultural age of clones and their effects on the kinetics of fusion, but not necessarily on the amount of fusion that occurred. Additional results from this study (Ross et al., 1973) also provided support for the existence of a required induction period before the beginning of fusion. They further discovered that as myxamoeba near encystment, they exhibit a diminished capacity to fuse which was suggested as being attributed to either an increasing density of cells, or an increasing number of cell generations. Transfer to fresh medium causes a de-differentiation of these cells to an immature state, and fusion competence can only be recovered through re-differentiation over time. Yemma and Stroh (1991) showed that a loss of induction results whenever cells become encysted. Thus it was concluded that induction of sexual competency is controlled by both a chemical factor and a necessity for membrane contact between cells before fusion can occur. Furthermore, the process of gametic fusion may also be under lysosomal control as cell lysis was prevalent in mixed liquid cultures of compatible mating types. Lysis was absent in mixed cultures that were immediately plated on solid agar, thus suggesting that normal contact between plasma membranes of compatible mating types may in some manner exert control on lysosomal activity (Ross et al., 1973). Exposure of myxamoeba to the chemical inducing substance causes a repression of the plasmodium forming, genes and activation of the gametic genes,

which produce those cell surface changes which confer recognition and fusion ability upon the myxamoeba. If successful fusion does not result, de-repression of the plasmodium-forming genes cannot take place and the organism will be subjected to lysosomal control and possible lysis.

Later on in 1973, Ross and Shipley postulated a seven-step sequence of interactions which result in sexual and somatic fusions in **D. iridis**. Mutual induction is initiated by a chemical substance which is released by all mating types that in turn leads to a de-repression of the genes that control fusion. The activation of these genes causes conformational and/or composition changes in the membrane surface, which allows for recognition of compatible mating types thus activating mechanisms which enable membranes to come into contact and fuse. It wasn't until the isolation of the inducer molecule, though, by Yemma and Stroh (1991) that all speculation as to the nature of this chemical substance was laid to rest. Once fusion occurs, molecular modification of the membrane surface occurs and prevents further fusion with other haploid cells. This modified membrane surface does, however, permit fusion to occur between zygotes and microplasmodia. Once successful sexual fusion has taken place, mature plasmodia begin to develop and the membrane surface is further modified to prevent heterokaryotic somatic fusions, but permits fusion between somatic cells of identical type. Proper execution of this sequence requires protein synthesis after each of the two gene de-repression

steps, and also to fuel the mechanisms which permit membrane contact and fusion. This actual mechanism of membrane contact and fusion is postulated as being lysosomally mediated. Treatment of cultures containing myxamoeba of compatible mating types with antibiotics and lysosomal stabilizers both before and after sexual fusion, produced results suggesting that protein synthesis is necessary for sexual fusion, but may not be for early somatic fusions (Ross and Shipley, 1973).

The existence of an inducer substance had been postulated, but it wasn't until the work of Yemma and Stroh (1991) that concrete evidence had been provided to support this theory. Prior to this discovery in 1991, the nearest anyone had come to isolating the inducer molecule was in 1977 when Youngman et al. were working with the myxomycete species **Physarum polycephalum**. Their study accomplished the separation of two amoebic cultures--one containing a dense population of myxamoeba and the other containing a sparse population of myxamoeba--by a pair of Nucleopore filters with a pore size of .05 microns. Nutrition for the myxamoeba was provided using both live and formalin-killed bacteria and this was done to insure that the possibility of induction by altered bacterial growth or by a substance released by bacteria was eliminated. Also, the size of the pores in the filters eliminated the possibility of diffusion of a myxamoeba through the filters since all haploid amoeba are larger than .05 microns in size. Results of these experiments did indicate that sparse amoebic cultures could be induced to

differentiate by dense amoebic cultures when the two were separated by a pair of .05 micron Nucleopore filters. If the dense culture was replaced by a culture of only bacteria, or by a culture containing amoeba from a cellular slime mold, induction of the sparse culture fails to occur. This evidence supported the hypothesis that induction is mediated by a diffusible chemical substance that is produced by a dense culture of myxamoeba. Results also indicated that this inducer substance possesses a short range of action as induction could not be produced if cultures were separated by more than two filters nor could it occur if the thickness of the agar media on which the cells were cultured was greater than 2 millimeters.

Before fusion competence can occur in a culture of myxamoeba, a critical cell density must be reached in each compatible mating type. Yemma and Perry (1985) determined this critical cell density to be 1 x 10 E+05 cells/milliliter. Thus when myxamoeba begin to mate after a critical cell density is attained, this is most likely a reaction to the accumulation of an inducer molecule that is released by the differentiating amoeba (Youngman et al., 1977; Pallotta et al., 1979). Shipley and Holt (1980, 1982) provided evidence in which cells that were unable to fuse in low density cultures could overcome this fusion incompetence through the action of an inducer material produced by cells of a critical density population. By modifying the methods of Youngman et al. (1977), Shipley and Holt (1982) suggested that

a critical cell density of 1 x 10 E+05 cells/milliliter produced maximum membrane fusion competence in mating compatible myxamoeba of **D. iridis**. They further suggested that myxamoeba of the myxomycete **Physarum polycephalum** exhibited this same maximal fusion competence at one-tenth the critical density required by **D. iridis**. Although Shipley and Holt postulated this critical cell density in 1982, it wasn't until 1985 that Yemma and Perry actually determined this to be true by verifying that mating competence is achieved during log phase of cultural growth when cells attain the density of 1 x 10 E+05 cells/milliliter, and was only possible when cells are in the G_1 phase of the cell cycle. The 1982 Shipley and Holt study also suggested that fusion competence could be achieved by individual mating types without interacting with compatible mating types. It is unknown whether variance of degree of competence in a population of myxamoeba is attributed to changes in the proportion of competent amoeba or in the level of individual cell competence in a population. Results of these experiments concludingly suggested that fusion competence is somehow dependent on continuously varying properties of the cells and further evidence alluded to the likelihood that once mating begins, fusion competence deteriorates rather quickly. This latter point was determined to be untrue as Yemma and Perry (1985) and again later Yemma and Stroh (1991) found that fusion competence is only lost when cells become encysted.

Building on the suggestion of the existence of an inducer molecule (Yemma and Therrien, 1974), Albert and Therrien (1985) hypothesized that induction occurs between all compatible mating strains of **D. iridis**, not just those studied in the literature. They also indicated that since mating type is a genetic representation of each individual strain, then the inducer molecule (referred to here as mating hormone) must be a unique genetic representation of each individual strain. These two hypotheses had been suggested before in reference to **Physarum polycephalum**, but were very preliminary regarding what was later to be suggested. Postulation that there existed a single receptor site to which all compatible mating hormones can bind to initiate induction lead to the assumption that the existing set of mating types evolved from a simpler set or if it was assumed that a single gene controls the mating hormone receptor site, then it is possible that a new mating type could evolve through modification of its existing genes. If indeed this sole receptor site was to recognize a diversity of mating hormones, then these hormones (inducer molecules) must exhibit a high degree of structural similarity. Continuing, in order to limit the amount of hormone self recognition and allow for diffusion of each respective mating hormone, each mating type should exhibit a comparably lower binding affinity and poorer activating characteristics for its own mating hormone. Finally, at extremely high concentrations of self-produced mating hormone, and primarily due to structural similarities

of mating hormones and receptor sites, the effects of induction between compatible mating types can be partially simulated through a self-recognition fusion cellular mechanism. But all characteristics associated with self-induction should also be associated with cross-induction. The major exception would be that most of the mating hormone receptor sites on the self-induced myxamoeba would be occupied by self-mating hormone and hence would be competitively inhibited to other possible inducers.

Although a mating (fusion) receptor has been postulated, evidence must be provided to support this claim. Evidence concerning the existence of cell surface receptors has been provided in reference to the cellular slime mold **Dictyostelium discoideum** (Gillette et al., 1974; Molday et al., 1976), but as of yet none has been provided in reference to the heterothallic myxomycetes. These two aforereferenced studies utilized the plasma membrane glycoprotein binding properties of concanavalin A to indicate the possible existence of this membrane surface receptor. This led Yemma and Soltis (1988) to hypothesize that the mating receptor on the surface of **D. iridis** was also a glycoprotein and therefore concanavalin A could also be used to locate this mating receptor. Culturing compatible mating types on media in the presence of concanavalin A revealed an almost complete absence of plasmodial formation, while culturing of these same mating type mixtures on media in the absence of concanavalin A exhibited nearly one hundred percent plasmodial formation.

These results indicated that fusion is blocked in the presence of concanavalin A, suggesting that this lectin must block fusion by binding to the membrane fusion (mating) receptor, which must be a glycoprotein since concanavalin A exhibits membrane glycoprotein binding properties. In that study it was stated that it is also very probable that this mating receptor is composed of alpha-mannopyranase and alpha-glucopyranase residues as concanavalin A preferentially binds these moieties.

Evidence has now been provided supporting the existence of both an inducer substance (or mating hormone as it is sometimes called) and a receptor for this inducer molecule which permits membrane fusion to occur and subsequent zygote and plasmodial formation to take place. The next step was the attempted isolation and characterization of both inducer and the mating receptor. Once the inducer substance is released into culture and either self- or cross-induction occurs, synthesis of the membrane receptors prerequisite to fusion and zygote formation follows shortly thereafter (Yemma and Stroh, 1991). Support for this required receptor site synthesis was provided by Yemma and Perry (1985), as they revealed that zygote formation in **D. iridis** was a time-dependent process. It is of utmost necessity that the cell cycle period for maturation be present in order for induction to occur, therefore a constant necessity exists for the production and presence of the inducer molecule (Yemma and Stroh, 1991). This previous study also presented high performance liquid

chromatography (HPLC) data which illustrated that the inducer molecule is labile and this lability is time dependent. In addition, increasing the concentration of the inducer molecule does not result in greater zygote formation as the determining factor in induction and zygote formation is cell maturity. Self-induced induction can only be produced at a critical cell density of 1 x 10 E+05 cells/milliliter (Yemma and Perry, 1985).

The purpose of this study was to obtain a purified plasma membrane isolation in the myxomycete **D. iridis** using the mating type HON 1-7A[2] (Albert and Therrien, 1985). Once purified plasma membrane fractions are isolated on both induced and uninduced myxamoeba of this mating type, HPLC analysis will examine the membrane protein profile and thus possibly isolate the inducible plasma membrane fusion receptor. A large portion of this study focuses on the isolation of the plasma membrane in **D. iridis** utilizing the technique of density gradient centrifugation. In 1977, Yemma and Selanik elucidated a method for rapid plasma membrane isolation of both myxamoeba and swarm cells of **D. iridis** using an aqueous two-phase polyethyleneglycol (PEG) polymer system. This method proved somewhat effective if only a rapid plasma membrane isolation was desired, but membrane yield and purity were low. Since the ultimate goal of this study was to isolate and possibly characterize the plasma membrane fusion receptor or mating factors, it was necessary to isolate the plasma membrane of **D. iridis**. This

had to be done by a technique whereby the plasma membrane would not be subjected to possible chemical alterations of its surface or membrane protein activity by substances such as, for example, PEG polymer. Initially, the PEG polymer method was designed to eliminate the need for isolation using expensive high speed centrifugation equipment which subjected myxamoeba cells to disruption or alteration of enzymatic activity, due to intense high speed gravitational forces. Although a successful gradient density plasma membrane isolation technique had not been developed for **D. iridis**, it has been successfully carried out on membranes of the myxomycete **P. polycephalum** (Barden et al., 1983; Reiskind and Aldrich, 1984). Modification of those isolation techniques used by Barden et al. (1983), served as the initial protocol upon which isolation and purification of **D. iridis** plasma membranes was based. The isolation technique utilized a sucrose gradient and high-speed refrigerated centrifugation. Treatment of the myxamoeba cells before the final plasma membrane isolation was nondisruptive, and required subjecting the cells to only mild salt solutions for washing and bacterial removal, followed by incubation in a buffered solution prior to loading onto the linear sucrose gradients. It proved advantageous to treat the plasma membranes of the cells under study with as few reagents as possible during the isolation. This eliminated the possibility of interaction of isolation reagents which could alter or remove plasma membrane constituents. Also, the

sensitivity of the HPLC regarding the analysis of membrane proteins is very high, and since the actual amount of isolated plasma membrane is not large, attempted removal of any isolation reagents after isolation of the plasma membrane, and before HPLC analysis, could result in the loss of the isolated membrane fractions during this attempted purification. The isolated membrane fractions can be localized by enzymatic assays which utilize respective marker enzymes for the plasma membrane, and also for those organelles which could possibly cause contamination of the isolated membrane fractions. Once highly purified membrane fractions are localized, examination of the plasma for the presence of a membrane fusion receptor or mating factors in induced myxamoeba isolated membrane fractions could be carried out.

CHAPTER II

Materials and Methods

Cell Culturing

Stock cultures of isolates of the myxomycete D. iridis were maintained on half-strength cornmeal agar slants in wide mouth screw-top culture vessels. Mating type HON 1-7A^2 was used exclusively in this study. The isolate was originally provided by Dr. O. R. Collins, Department of Botany, University of California, Berkeley, and subsequently maintained for many years by Dr. John J. Yemma, Department of Biology, Youngstown State University and his graduate students.

Solid Media Culture Techniques

Half-strength cornmeal agar media was prepared by combining 8.5 grams of Difco Cornmeal Agar and 8.0 grams of Difco Bacto-Agar in one liter of distilled water. The medium was prepared and then autoclaved at 121° Celsius and 15 P. S. I. for fifteen minutes before being dispensed into sterile petri dishes in 10 milliliter aliquots (Collins, 1963; Yemma et al., 1974). Once these plates cooled and the agar sufficiently solidified, they were refrigerated until time of use to prevent fungal contamination. When plating of the experimental organism onto the medium was to occur, sleeves of half-strength cornmeal agar plates were removed from refrigeration and allowed to warm to room temperature. Escherichia coli bacterium was inoculated to the medium prior

to plating of **D. iridis** in order to provide a nutrient source for the growing cell population. All platings were carried out in a laminar flow hood in order to insure strictly sterile conditions. Plates were left in the laminar flow hood for 24 hours after inoculation with E. coli to ensure growth of a uniform bacterial lawn. HON 1-7A^2 cells were then transferred to the surface of the medium containing the bacterial lawn. Transfer of cells was also carried out in the sterile environment of a laminar flow hood. Plating was accomplished by the transfer of an agar block (approximately 2 centimeters square) from a primary culture tube of stock culture, or as the case may be from a half-strength cornmeal agar plate that was confluent with encysted myxamoeba of the HON 1-7A^2 isolate of **D. iridis**. Once the plates became confluent with myxamoeba, they were used to make additional transfers. Transfer of agar blocks from stock plates containing encysted cells, or from stock plates prepared by the transfer of spores from culture tube slant stock cultures, was always done in a laminar flow hood under sterile conditions. An Olympus CK2 inverted microscope was used in all cases to view and monitor growing cells. Once the stock plate was determined to be contaminant-free, it was placed back in the laminar flow hood where it was unsealed by removal of the external parafilm layer. Subsequent plating of myxamoeba to additional plates containing bacteria involved the transfer of 2 centimeter square agar blocks containing HON 1-7A^2 cells, by use of a sterile spatula. Each plate was then

properly labelled on the bottom with the type of isolate (HON 1-7A'), the individual's name, and the date on which the plating occurred. Daily contamination checks were conducted on the plates using an inverted microscope. Contaminated plates were quickly discarded. Replating of cells occurred every week to ten days from a contaminant-free plate, with the previously transferred plate serving as the stock plate.

Liquid Culture Techniques

Liquid cell cultures, when needed, were prepared in both small and large spin cultures. For the small spin cultures, cells were taken from three to five plates of previously plated half-strength cornmeal agar plates, while the large liquid cultures required cells from ten to fifteen plates. Cells were removed from these plates by the addition of a few milliliters of ultrapure millipore water to each plate using a sterile disposable pasteur pipette and then removing the cells from the agar surface by the repeated forced expelling action of water that had been drawn up into the pipette out onto the agar surface. Cells could also be removed from the surface by the scraping action of a rubber policeman. Once the cells had been forced from the agar surface of each plate, they were transferred to the small spin cultures using a sterile disposable pasteur pipette and to the large spin cultures by pouring the liquid contents of each plate into a small glass funnel containing a piece of cheesecloth (to filter out agar pieces). After addition of the cells, E. coli was supplied as a nutrient source with one slant of dilute

bacterium being added to the small spin cultures and three to four slants to the large spin cultures. The cultures were then diluted further by the addition of ultrapure millipore water until the solution was nearly clear. Aeration was provided utilizing magnetic stir bars. Liquid spin cultures allowed cells to be cultured in the large quantities necessary for the isolation of plasma membrane in large volume. Small spin cultures were utilized primarily for uninduced cell populations, since the cellular densities of these small cultures did not approach those of the large. The latter were used for harvesting induced populations. Cell growth in liquid cultures was monitored by taking daily cell counts using a hemacytometer. It is interesting to note that on occasion there was a buildup of debris along the bottom of cultures, and on magnetic stirrers in both small and large spin cultures. This was thought to be attributed to build-up of bacteria or of bacterial breakdown products. A search of the literature, however, suggested that once cells become induced in liquid culture, if membrane fusion or membrane contact between compatible mating types did not occur within two to three hours of induction (Ross et al., 1973), then activation of a lysosomal mechanism would occur and the cells would consequently lyse.

HON 1-7A' Isolate Cell Harvesting

To adequately isolate the plasma membrane from cells of the HON 1-7A' isolates, it was necessary to collect a large number of bacteria-free cells from cultures in both the

induced and uninduced forms. Therefore, in order to more accurately separate induced from non-induced cells, cell counts had to be made since cell density must be 1 x 10 E+05 cells/milliliter in order for induction to occur, and below this density no induction occurred (Yemma and Perry, 1985). Counts were made on cells contained in test tubes with a cellular density equivalent to that of the culture. Six tubes were counted and a daily average cell count was determined to provide greater accuracy. To prepare the tubes, cells were collected by the technique described earlier, whereby cells were forcibly drawn off the surface of the agar and transferred to spin cultures. In this preparation, cells were collected from solid agar plates in this manner (usually 10 to 15 plates) and added to an acid washed, millipore water rinsed 250 milliliter beaker. The cell-containing solution was then decanted into six 40 milliliter thick walled conical glass centrifuge tubes until the volume of each tube was approximately equal. The cells were then concentrated by being spun down at 1000 R. P. M.'s in an IEC HN-SII swinging bucket centrifuge for ten minutes. Any bacteria present remained in the supernatant. Upon completion of centrifugation, the pelleted cells were pooled into one of the centrifuge tubes and a cell count was taken by use of a hemacytometer. Once the cell count was determined, the cells were diluted with ultrapure millipore water and a fixed number of cells was transferred into each of six culture tubes with the dilution factor being mathematically determined. The

tubes were then numbered and placed in a test tube rack which had already been secured in an oscillating water bath. The tubes were then shaken at 50 to 70 oscillations per minute at room temperature and daily cell counts were recorded after the tubes had been transferred to a laminar flow hood to insure sterile procedure. Two counts were taken for each tube. Cell counts were taken daily approximately 24 hours apart, until proper numbers were recorded. Encysted HON 1-7A' isolates were replated onto fresh bacteria-containing cornmeal agar, since these cells lose their mating competency (Yemma and Stroh, 1991) and thus are uninduced immature forms that must be grown over a period of several days until they become competent enough to undergo induction. This requires that a large number of cells, at least 1 x 10 E+05 cells/milliliter (Yemma and Stroh, 1991), be collected from agar medium plates in order to execute a satisfactory plasma membrane isolation. An isolation of induced cells was carried out initially. Prior to collection of the plasma membrane, isolates containing half-strength cornmeal agar plates were checked daily for both contamination and progress of growth. Approximately five to seven days after cells had been plated to fresh agar, they were removed from the surface agar by the techniques previously described, and filtered through a layer of cheesecloth lining a glass funnel into an acid washed one liter Erlenmeyer flask. The intent here was to collect induced cells as Yemma and Stroh (1991) also showed that encysted cells take approximately five to seven days to regain

their fusion competency by attaining the required cell density of 1 x 10 E+05 cells/milliliter. Since induced cells are in a much greater density than uninduced cells, they were chosen to initially attempt the plasma membrane isolation technique.

Cells of the myxomycete **D. iridis** can only mate when the cell population of each mating type reaches a critical density, or when a mating type population of critical density is separated from a compatible mating type population, below critical cell density. In both cases, cells of each mating type (isolate) were able to fuse with induced cells of compatible mating types. Work done by Yemma and Stroh (1991) serves to emphasize the importance of attainment of the critical cell density of 1 x 10 E+05 cells/milliliter. Their work also showed that fusion between cells that had not gained induction competency through either of the two aforementioned reasons, did not occur. In this study, attainment of the 1 x 10 E+05 cells/milliliter critical cell density was the method used for determining fusion competency. Insight regarding the process of induction and how it may occur between different mating type populations is of major consideration in the present study.

Plasma Membrane Isolation of HON 1-7A' Cells

Cells were collected from forty to sixty plates all initially plated on the same date. Pooled cells were then decanted into six 40 milliliter thick-walled conical glass centrifuge tubes. Tubes were centrifuged for 10 minutes at

1000 R. P. M.'s in an IEC HN-SII swinging bucket centrifuge, in order to remove any bacteria that may have been present. The pelleted cells were suspended by the addition of approximately twenty milliliters of ultrapure millipore water to each tube and then repelleted in the same manner as the previous step. This step was repeated until the supernatant layer was no longer turbid (usually accomplished in three or four spins), thus indicating the removal of any bacterial contamination from the pelleted cells. It is also important to note that if cells grew vigorously, the remaining cell suspension was relatively bacteria-free due to the ingestion of bacteria by growing cells. The bacteria-free cells were again pooled into one tube and then transferred to a 50 milliliter round bottom polyallomer centrifuge tube. From this point on, the plasma membrane isolation technique was carried out. Bacteria-free pooled cells were initially pelleted using an IEC BA-20 refrigerated centrifuge with a fixed angle rotor at 2000 R. P. M. for ten minutes. The supernatant was drawn off, exercising extreme care not to disrupt the pellet. The pelleted cells were further washed by being twice resuspended in 10 milliliters of cold .14 M NaCl each time followed by centrifugation and removal of the supernatant. The cleansed pellet was then further suspended in 15 milliliters of room temperature homogenization buffer consisting of 1 mM ZnCl and 10 mM Tris-HCl (pH 8.0) in a 1:1 ratio. The cells remained suspended at room temperature for 15 minutes at which time they were cooled to 4^{0} Celsius by

partial immersion of the polyallomer centrifuge tube into an icewater bath in a one liter beaker. All remaining steps of the isolation were carried out at this temperature. Once the cell suspension was cooled to the desired temperature, the cells were transferred to a 30 milliliter polyallomer oak ridge ultracentrifuge tube for the disruption of whole cells. Disruption occurred upon exposure to a constant sonication frequency for 5 to 6 seconds produced by a Branson Sonifier 250 fitted with an Ultrasonics Converter. Successful sonication was determined by microscopic examination. Sonically produced cellular fragments were then concentrated into a pellet by centrifugation at 14,000 R. P. M. for 15 minutes in a Beckman L7-35 Refrigerated Ultracentrifuge using a fixed angle rotor type 42.1, and precooling the centrifugation chamber to 4° Celsius along with the buildup of at least a 10 micron vacuum. The supernatant layer was again drawn off with the resulting pellet suspended in 7.5 milliliters of a 10% (w/v) sucrose solution. The cell fragment-containing sucrose suspension was further disrupted by a one second sonication (as previously described). The further disrupted suspension was loaded onto 20% to 50% (w/v) linear sucrose gradients in 6 milliliter aliquots per gradient. Linear sucrose gradients were prepared 24 hours prior to centrifugation in a 30 milliliter oak ridge polyallomer ultracentrifuge tube using 10 milliliter graduated borscilate glass pipettes and vacuum pipette bulbs to carefully run each successive aliquot layer down the side of

the tube to avoid any major disruption of the layers already present in the tube. Preparation of the linear sucrose gradient 24 hours prior to centrifugation was done in order to allow each individual layer to settle after possible mixing of the layers during the pipetting process. Overnight refrigeration of the formed gradients provided a better separation of the four individual layers, which in turn contributed to a cleaner separation of membrane components upon centrifugation the following day. The sonicated 10% (w/v) sucrose suspension was loaded onto the 20% to 50% (w/v) linear sucrose gradient in a 5 milliliter aliquot, and centrifugation was carried out at 26,000 R. P. M. for 2 hours. Once the membrane components had been separated, fractions were collected in small aliquots and subjected to enzymatic assays in order to determine the location(s) of the appropriate plasma membrane fraction(s). To accomplish this, a BIO-RAD model 2110 fraction collector was used. Linked to this apparatus was a New Brunswick Scientific peristaltic pump (model M1062), and a Powerstat 10 amp variable output (10-40 volts) autotransformer (type 3PN116B). Tubing (which served as the vehicle of transport for the liquid fractions) ran from the centrifuge tube to the dispensing pump, and finally to the fraction collector which permitted approximately 2 milliliter aliquots to be collected in 4.0 milliliter Corning self-standing disposable sterile cryogenic vials. The dispensing pump was connected to a variable output autotransformer to help control the rate at which the fractions were pumped

through the tubing and collected. Determination of the proper pumping rate was found through trial and error. The free end of tygon tubing was placed in a solution of sterile HPLC water in an acid washed beaker, and the variable output autotransformer was adjusted until a steady flow of solution was visible in the tubing. If a steady flow rate could not be produced, it was sometimes necessary to adjust the force with which the tubing was contracted in the dispensing pump by either loosening the tension applied on the tubing in each contraction, or tightening the tension and thus making each contraction more forceful. The trial and error process to determine the output necessary to maintain this initiated rate of flow was repeated until a suitable setting was discovered. When a steady flow rate had been produced, determination of the most effective method for consistent collection of successive 2 milliliter aliquots was done in order to attain approximately repeatable collection results with each respective isolation. It was found that fraction collection done on the basis of time was most suitable in this study as oppopsed to collection by drop count. The desired volume of each fraction was again found by trial and error. The volumes of the resulting fractions collected by time were very consistent with slight variation attributed to the sometimes errant accuracy of the rubber drop former. To rectify this problem, a 200 microliter capacity Drummond MICRODISPENSER glass replacement tube was cut to the proper length and securely placed over the outside of the drop former

in such a manner that accuracy of the resulting drops was significantly improved, while at the same time this modification did not interfere with the automatic advance of collection vials by the fraction collector. Once all the proper settings were determined for consistent collection of all respective fractions, the free end of the tubing was removed from the HPLC water in the beaker and the remaining water was allowed to be pumped out of the tubing and into a collection beaker for disposal. Once the fractionation system had been set, the power was shut off until centrifugation was completed and the respective tubes were prepared for fractionation. Upon completion of centrifugation, it was important that the rotor containing the sucrose gradient tubes be removed from the refrigerated centrifugation chamber as soon as possible as prolonged exposure to the temperature in the centrifugation chamber without spinning could result in partial solidification of the gradients in the tube and therefore be disruptive to the fractionation process. The free end of the Tygon tubing used in fractionation was then very carefully slid down the inside surface of the centrifuge tube until the tip of the tubing reached the bottom of the tube. At this point, the fraction collector and dispensing pump were turned on, but not the variable output autotransformer as this served as the trigger switch for activation of the pump. After verification of all settings and ensuring that the tubing was securely fastened in the drop former of the fraction collector, the variable output

autotransformer was switched on thereby activating the dispensing pump and initiating movement of the sucrose gradient solution into the tubing. Once the flow of liquid was visibly shown to pass through the tubing to the drop former of the fraction collector, the fraction collector was switched to its running mode and the tubes were automatically advanced as the set time expired for filling each respective tube. Fractionation was completed when the contents of the centrifuge tube had all been pumped out as indicated by visual observation. All collected fractions were then kept cold. The carousel of the fraction collector was then reset and filled with freshly labelled cryogenic tubes. The Tygon tubing was then flushed out with several milliliters of HPLC water to remove any remaining residue from the just completed fractionation by activation of the peristaltic pump through the switching on of the autotransformer. Once the tubing was flushed and all remaining HPLC water removed from it, fractionation of a second tube was executed in the same manner as previously described. Upon completion of collection of all fractions, the tubing was again flushed with HPLC water to prevent buildup of residual components. Cryogenic vials containing the respective fractions were kept cold until they could be transferred for storage in liquid nitrogen in a Taylor-Wharton 35 HC liquid nitrogen storage container until enzymatic assays could be performed.

Enzymatic Assays

5'-Nucleotidase Assay

5'-nucleotidase served as the principal plasma membrane marker in this study. Activity of the plasma membrane marker enzyme was determined through measurement of the amount of inorganic phosphate that was liberated by the marker enzyme substrate. This activity was described by Evans (1978) and the released inorganic phosphate was quantified by the method of Chen et al. (1956). The presence of 5'-nucleotidase was determined by measuring the amount of inorganic phosphate that was released through its cleavage from the substrate 5'-adenosine monophosphate. The substrate which was obtained from Sigma Chemical Co. (A1752, type II) was one of the components of the final incubation mixture consisting of 100 mM KCl, 10 mM magnesium chloride, 50 mM Tris-HCl (pH 7.4), 10 mM sodium potassium tartrate, and 5 mM 5'-adenosine monophosphate (AMP) (Barden et al., 1983). The mixture was allowed to incubate for at least 10 to 15 minutes, at which time .5 milliliter aliquots were transferred to fresh 13 x 100 millimeter disposable culture tubes (VWR Scientific Co.) using a 1 milliliter capacity Oxford Sampler micropipettor and corresponding tip. Each tube was appropriately labelled with all information present on the cryogenic vial containing the corresponding isolated fraction. After removing the collected fractions from liquid nitrogen storage, the cryogenic vials were thawed by being placed in a container filled with cold tap water. The temperature of the water allowed the vials to

thaw while at the same time keeping the temperature of their contents from rising above the critical temperature of 4 Celsius. Once the solution in the vials had thawed, ice was added to the tap water to keep the overall temperature of the surrounding water below that of the critical temperature. Next, 50 microliters of each respective fraction was added to the incubation mixture in the correspondingly labelled culture tube and upon completion of the addition of all fractions, the culture tubes were incubated for 15 minutes at 26 Celsius in a Precision Scientific Forced Air Mechanical Convection Oven (model 605). During this incubation period, the fraction-containing cryogenic vials were returned to storage in liquid nitrogen. At the end of the incubation period, the culture tubes were removed from the oven and the 5'-nucleotidase-inorganic phosphate release reaction was terminated by the addition of 50 microliters of 20% (w/v) trichloroacetic acid. (If a white precipitate formed in any of the tubes, these tubes were to be centrifuged at 2000 R.P.M. for 5 minutes in an IEC HN-SII swinging bucket centrifuge followed by the removal and saving of the supernatant layer and discarding of the pellet. The saved supernatant layer would be utilized throughout the remainder of the assay.) The volume of the clear mixture in each culture tube was brought up to 4.0 milliliters by the addition of distilled ultrapure millipore water. This was followed by the addition of 4.0 milliliters of Reagent C or the coloring agent using a sterile graduated glass pipette. This reagent must be prepared fresh daily by

mixing 6 N sulfuric acid, distilled ultrapure millipore water, 2.5% (w/v) ammonium molybdate, and 10% (w/v) ascorbic acid in a 1:2:1:1 ratio. A yellowish color is characteristic of this mixture and refrigeration was necessary in order to retain this color. Upon completion of the addition of Reagent C, each tube was sealed with parafilm and shaken by repeated inversion of the sealed tube to insure adequate mixture of the contents inside. These tubes were then returned to the forced air oven where they were left to incubate for 1.5 to 2 hours at 37° Celsius. To insure that all reagents were active and that the reaction was proceeding properly, a positive control was run with each 5'-nucleotidase assay. The control was prepared by the substitution of a few micrograms of 5'-nucleotidase (which was obtained from the Sigma Chemical Co.,N-4005) in place of 50 microliters of a respective membrane fraction just prior to the 15-minute incubation period at 26° Celsius. After the specified incubation period at 37° Celsius, the tubes were removed from the oven and allowed to cool for a few minutes before absorption readings were taken on a spectrophotometer. Once the tubes had cooled down, the parafilm seals were removed from each one. Measurement of the amount of inorganic phosphate released was done utilizing the fact that a color change (yellow to blue) will occur if the 5'-nucleotidase reaction is positive. Since the amount of purified plasma membrane was so minute, this color change was very subtle and required, for the sake of accuracy, measurement against a distilled water blank and

comparison to a previously determined calibration curve. The coloring agent, Reagent C, maximally absorbed in the ultraviolet portion of the spectrum at 820 nanometers, therefore a calibration curve was determined using three standard amounts of the enzyme 5'-nucleotidase (i. e. 5, 10, and 20 microliters) in place of respective membrane fractions, to prepare a linear curve against which all the collected fractions were compared. The absorbance readings of all samples (including the calibration standards) were taken on a Perkin Elmer Lambda 2 UV/VIS spectrophotometer which was connected to an Epson Equity III+ computer. Before any samples were read, the spectrophotometer was autozeroed at 820 nanometers using a distilled water blank. From this point on, both calibration standards and collected isolation fractions were given a sample identity corresponding to either their enzyme concentration value in the case of the standards or to their respective numerical identifications corresponding to the area from which the fraction was collected in the linear sucrose gradient. Each respective sample was decanted into a UV disposable cuvette and the exterior of the cuvette was wiped clean to prevent errant absorption values which could be caused by extraneous markings. The clean cuvette was placed into its respective holder in the spectrophotometer and the absorption of the sample was determined producing ordinate and concentration values which were extrapolated from the calibration curve. The fractions from which the highest ordinate and concentration values were recorded for the

quantification of the inorganic phosphate released were determined to have the highest concentration of 5'-nucleotidase, thus indicating the location of the isolated plasma membrane fragments. The plasma membrane-containing fractions were then assayed for microsomal, lysosomal, and mitochondrial contamination utilizing the characteristic marker enzymes glucose-6-phosphatase, acid phosphatase, and cytochrome oxidase, respectively.

Glucose-6-Phosphatase Assay

Glucose-6-phosphatase is the marker enzyme for the smooth endoplasmic reticulum and its enzymatic activity was measured in a manner similar to that of 5'-nucleotidase. Because of its cellular concentration, it is usually implicated in contamination of isolated plasma membrane fractions. Glucose-6-phosphatase activity was assayed by measuring the amount of inorganic phosphate that was released from the substrate glucose-6-phosphate (Sigma Chemical Co., G-7250). Assay of this activity utilized the method of Swanson (1977) while measurement of the amount of released inorganic phosphate was shown again according to the method of Chen et al. (1956). The final 1 milliliter reaction mixture contained 100 micromolar sodium acetate (pH 6.2), 4 mM EDTA, 0.5 mM sodium fluoride, 2 mM glucose-6-phosphate, and 50 microliters of one of the respective plasma membrane fractions. This assay was carried out in the same manner as described in the 5'-nucleotidase assay including the culture tube incubation vessels, pipetting equipment and techniques, and all

incubation setups and procedures. After a 15 minute incubation period at room temperature, the glucose-6-phosphatase reaction was stopped with 250 microliters of 50% (w/v) trichloroacetic acid. The remainder of this assay concerning the measurement of the inorganic phosphate released followed exactly those steps outlined in the 5'-nucleotidase assay including use of Reagent C as the coloring agent and absorption readings taken at 820 nanometers. A calibration curve was also prepared as was a positive control each substituting the enzyme glucose-6-phosphatase (Sigma Chemical Co., G-5758) in place of a respective plasma membrane fraction. If any membrane fraction revealed a significant concentration of inorganic phosphate present, this indicated the presence of glucose-6-phosphatase and therefore, microsomal contamination.

Acid Phosphatase Assay

Acid phosphatase is the marker enzyme for the lysosome. The assay protocol followed in this study was described by Barnett and Heath (1977). This assay was carried out in the same manner as the two assays already described in this chapter generally utilizing the same type of equipment and techniques. Before this assay was run, the membrane fractions were twice frozen in liquid nitrogen and then thawed to insure lysosomal breakage. The final incubation mixture was comprised of 1.2 milliliters of 5 mM sodium acetate which was adjusted to pH 5.0 with dilute acetic acid, 0.5 milliliters of 5 mM p-nitrophenyl phosphate, and 0.3 milliliters of a

respective membrane fraction. Following a 20 minute room temperature incubation, the reaction was terminated with 100 microliters of a solution containing 1 M Tris, 1 M potassium phosphate, and 0.25 M EDTA (pH 10.5) in a 1:1:1 ratio. Bis (p-nitrophenyl) phosphate (Sigma Chemical Co., N-1256) served as the substrate for the reaction. A positive control was run substituting 0.3 milliliters of a solution of o-nitrophenol (which was also obtained from Sigma Chemical Co., N-9256) at a concentration of 92.7 micrograms/milliliter for the same volume of membrane fraction. Absorbance was again measured with a Perkin Elmer Lambda 2 UV/VIS spectrophotometer but this time at 420 nanometers against a distilled water blank. The solution which terminates this reaction was high in phosphate concentration in order to inhibit alkaline phosphatase activity thus preventing interference with the acid phosphatase activity. Any membrane fractions which exhibited significantly high concentrations of acid phosphatase when compared to calibration standards were considered to be contaminated by lysosomal components and would thus prove inappropriate for further analysis.

Cytochrome Oxidase Assay

Mitochondrial contamination, as shown by the presence of the marker enzyme cytochrome oxidase, was the final assay to test for the presence of a contaminant-free plasma membrane fraction. This procedure followed that as described by Cooperstein and Lazarow (1951) in which 15 milliliters of a .08 M solution of cytochrome c (Sigma Chemical Co., C-2506,

type III) was reduced by the addition of 100 microliters of a freshly prepared solution of 1.2 M sodium hydrosulfite. This reduced cytochrome c solution was combined with 15.0 milliliters of a 60 mM phosphate buffer solution (pH 7.6) and 3.0 milliliter aliquots were pipetted into clean sterile culture tubes. Quantification of this enzyme was based on measurement of the rate of decrease in absorbance at 550 nanometers again using a Perkin Elmer Lambda 2 UV/VIS spectrophotometer. This procedure differed from that of the other three assays in that the membrane fraction was not added until it was time to read the respective sample on the spectrophotometer. When it was time for the absorbance of a particular plasma membrane fraction to be determined, 50.0 microliters of the fraction was added to one of the respective tubes containing a 3.0 milliliter aliquot of the reduced cytochrome c-phosphate buffer solution. A small parafilm square was placed over the opening of the tube and the tube was inverted to mix the contents. The contents of the culture tube were immediately transferred to a UV disposable cuvette, the exterior wiped clean, and an absorbance reading taken at 550 nanometers against a distilled water blank. Absorbance readings were taken at 30.0 second intervals over a 3.0 minute period after which a few crystals of potassium ferricyanide were added to the solution in the cuvette, the contents mixed (as previously mentioned by inversion), and absorbance readings again taken at 30.0-second intervals until a decrease in absorbance no longer occurred. This process of absorbance

determinations proved unorthodox in the calibration of a standard curve, therefore membrane fractions which initially produced significantly high absorbance values were taken to be positive for cytochrome oxidase activity and thus contaminated with mitochondrial fragments. Again a positive control was run and this time the membrane fraction was replaced by the enzyme cytochrome oxidase which was obtained from Sigma Chemical Co. (C-5771).

All membrane fractions determined to be free from these three contaminants were capable of undergoing HPLC analysis. For each of these assays (especially 5'-nucleotidase and glucose-6-phosphatase), glassware was always washed with a dichromic acid solution and rinsed thoroughly with ultrapure millipore water. This prevented any interference in the assay reactions from phosphate buildup on the glassware surface attributed to frequent cleaning with a phosphorous-based detergent. Also, pH adjustments on all specified solutions were done using dilute solutions of hydrochloric acid or dilute solutions of sodium hydroxide. These adjustments were monitored using an Orion Research Expandable Ion Analyzer EA 920 pH meter with a stir plate and stirring bar to sufficiently mix the contents of the solution.

High Performance Liquid Chromatography (HPLC) Analysis

HPLC analysis was done on the enzymatic assay-determined, isolated purified membrane fractions of induced and uninduced cells. To check for the possibility of sucrose interference in HPLC analysis, a spectrophotometric scan of a

.001 M sucrose solution was run from 800.0-190.0 nanometers using a Perkin Elmer Lambda 2 UV/VIS spectrophotometer. Results of this scan indicated that sucrose maximally absorbs at 209.0 nanometers and this was verified by a technical service representative of Sigma Chemical Co., from which the sucrose was obtained. The plasma membrane fusion receptor has been postulated as being a glycoprotein as shown by Yemma and Soltis (1988) through their use of the membrane glycoprotein-binding substance concanavalin A, in blocking plasma membrane fusion between cells of compatible mating type. Gennis (1989), among others, indicated that membrane proteins, glycoprotein receptors in particular, absorb maximally at 280.0 nanometers; therefore, by definition, attached sucrose molecules would not interfere with the absorption of eluted membrane proteins. Barden et al. (1983) avoided interference by sucrose molecules through dilution of the membrane fractions with water and then removal of the soluble sucrose by centrifugation. This study, however, demonstrated that sucrose did not cause interference in enzymatic assays or HPLC analysis. Therefore, its removal was deemed to be unnecessary, and thus prevented a loss of membrane fraction through sucrose removal.

Column type and mobile phase determination for the separation of plasma membrane proteins was suggested by Regnier (1984), but the actual parameters chosen were taken from Yemma and Green (1988) based on their analysis of methionine and leucine enkephalins. HPLC analysis was

done on a Perkin Elmer Series 400 Liquid chromatograph equipped with an ISS-100 autosampler, an LC-100 column oven, and an LC-75 variable wavelength spectrophotometric detector which was connected through an interface to a model 7500 professional computer and model PR 310 printer for recording peaks, plotting curves, and integrating the area under these curves. Separation was done using a C-18 Aquapore RP-300 reverse phase column from Pierce Chemical Co. HPLC grade acetonitrile and .02 M phosphate buffer, pH 6.0 (made from sodium phosphate monobasic and sodium phosphate dibasic) served as the mobile phase solutions while ultrapure millipore water served to flush the column clean before each new run. Each of these solutions was individually filtered by vacuum filtration through a filtration apparatus from Pierce Chemical Co. which was fitted with a Filterpure 47 millimeter, 0.45 micron Nylon-66 filter membrane also obtained from Pierce. Each filtered solution was transferred from the filtration collection container to an acid washed one-liter vessel into which was placed the respective lines for programming the pumping of solutions through the column. The solutions were then deoxygenated by bubbling in pure helium for five minutes after which the column lines were purged by activating the pump and removing 5 milliliters of solution from each set of lines. The pump was then programmed for a 10-minute equilibration step with pure acetonitrile followed by the delivery of the mobile phase in a linear gradient of 100% acetonitrile and 0% .02 M phosphate buffer (pH 6.0) to 50%

acetonitrile and 50% .02 M phosphate buffer (pH 6.0) over a 20-minute time period. A 10-minute water wash flushed the column clean and the sequence was repeated until all samples were run. The rate at which these solutions were pumped through the column to provide optimum separation of membrane proteins was determined to be 1.0 milliliters/minute. The pump was activated and the flow rate gradually increased until the desired flow rate was reached thus indicating the beginning of the equilibration step. While the column was equilibrating, sample preparation took place.

These membrane fractions which had been determined to be suitable for HPLC analysis were removed from liquid nitrogen storage and thawed by placement in a beaker containing cold water. Once thawed, a Perkin Elmer 2.0 milliliter crimp style glass vial was filled with approximately 0.4 milliliters each of acetonitrile and .02 M phosphate buffer (pH 6.0) and then the remaining capacity filled with thawed membrane fraction solution. Each solution was removed from its original container using a sterile syringe fitted with a sterile canula. Once solution had been drawn up into the syringe, the canula was removed and replaced by a 0.45 micron HPLC certified ACRO LC13 disposable filter and each solution was forced through one of these filters and into the sample vial in their desired volumes. The vial was capped with a Perkin Elmer Chromacol Ltd. 11 millimeter aluminum cap using a Wheaton crimper. The vial was labelled and kept at 4° Celsius until the sample was ready for injection. The autosampler was

programmed with the appropriate run time and injection volume (20 microliters in this case) until it was ready for injection. Once the equilibration step was completed and the pump display indicated 'PUMP READY', the sample was agitated by inverting to insure sufficient mixing of contents, and placed into position in the sample tray of the autosampler. The spectrophotometer had already been set at 280.0 nanometers and the computer programmed to receive data before any solutions or samples had been prepared. Sample injection and delivery of the mobile phase was initiated by activation of the autosampler. Immediately following sample injection, the sample vial was removed from the autosampler and returned to refrigeration at 4° Celsius in case a repeat run was necessary. This sequence was repeated with the running of each respective sample. The mobile phase and flush solutions were filtered daily while bleeding of the lines occurred at least weekly.

CHAPTER III

Results

Hon 1-7 A cells used throughout this study were cultured on half-strength cornmeal agar plates. In order to insure purity of the cell line, cultures were monitored for contamination using an inverted light microscope. The presence of any hyphae on the surface or within the agar indicated contamination and those plates were discarded.

Cell counts were obtained using an AO bright line hemacytometer, cell counter, and an Olympus phase contrast microscope. All counts were done under 40X magnification. As previously stated in the Materials and Methods section, cell count preparations were carried out using test tubes containing an initial fixed cell number. One drop of solution was taken from each 15 milliliter tube containing a precisely measured volume previously removed from a culture flask. Two counts were taken from each. Cell counts were necessary in order to chart the progress of cell growth and thus to note the approximate time period required for a cell population to attain the required density indicating an induced state population, or uninduced as the case may be (Yemma and Stroh, 1991). Since a dense population of cells was needed in order to provided a high plasma membrane yield as required in this study, specifically in the case of induced cells, a method which could satisfy this requirement had to be developed. An induced population, it is postulated, would

contain plasma membrane receptors or mating factors which could be analyzed. The primary isolation of plasma membranes, it was found, required a pure pellet of cells thus eliminating the possibility of any significant interference attributed to anything other than membrane molecules during subsequent assay of the plasma membrane marker enzyme. It was also found that the gradient density centrifugation technique presented, produced highly repeatable enzymatic assay results attesting to the purity of the isolated membrane fractions. It is also important to note that when sucrose gradients were freshly prepared, layered, and then refrigerated twelve to twenty-four hours before the isolation was to take place, improved results were obtained. Once the membrane preparations were loaded onto the sucrose gradients and centrifuged, it was of the utmost importance that the gradient be free from disturbances of any type other than those required to execute proper fractionation of the migrated preparation. The fractionation procedure previously described in the Materials and Methods chapter resulted in minimal disruption of the sucrose gradients and the elimination of the loss of any potential membrane-containing fraction(s) attributable to leakage. The fractionation process also produced highly repeatable enzymatic assay results used in determining the location of the major plasma membrane band as shown in Table 1. The repeatability of the resulting data attest to the efficiency and reliability of our technique.

5'-nucleotidase is the marker enzyme for the plasma

membrane. As mentioned previously, the 5'-nucleotidase assay requires the removal of inorganic phosphate from the substrate 5'-AMP by the plasma membrane marker enzyme 5'-nucleotidase. The amount of cleaved inorganic phosphate is then quantified by spectrophotometric assay, carried out at a wavelength of 820.0 nanometers. Quantification was carried out by the determination of the concentration of cleaved inorganic phosphate that reacts with the ascorbic acid coloring reagent to produce a bluish color change. The greater the amount of cleaved inorganic phosphate, the deeper the blue color produced by its reaction with ascorbic acid (thus the more positive the reaction) and therefore the greater its respective ordinate value and relative concentration value when extrapolated to a standard calibration curve as shown in Figure 2. 5'-nucleotidase standards were prepared from an enzymatic standard obtained from Sigma Chemical Co. (product no. N-4005, grade III). This product contained 100 units and weighed less than 1 gram. By definition there are 123 units/milligram of solid as indicated on the product vial, with a unit being defined as "the amount of lyophilized powder that will hydrolyze 1.0 micromole of inorganic phosphate from adenosine-5'-monophosphate per minute at pH 9.0 at 37° C." The assay was used in order to determine the purity of the isolated fractions. The location of the plasma membrane fractions utilizing this marker enzyme assay, exhibited the data presented in Table 1. As shown by these results, 5'-nucleotidase activity was found in fractions collected slightly below the middle of the linear sucrose gradient in

Table 1. Spectrophotometric Plasma Membrane 5'-Nucleotidase Assay Results.

5'-Nucleotidase Assay Results

Spectrophotometric Plasma Membrane Localization

Sample ** I. D.	Ordinate * Value	Concentration (g/ml)
control	.5258	9.68 E-08
5(a)	.0538	9.92 E-09
5(b)	.0566	1.04 E-08
8(a)	.0541	9.96 E-09
5(c)	.0538	9.92 E-09
3	.0745	1.37 E-08
4	.0742	1.37 E-08
5(d)	.0615	1.13 E-08
8(b)	.0559	1.03 E-08

** Membrane fraction values determined to be statistically significant from those of non-membrane fractions to 10E-06.

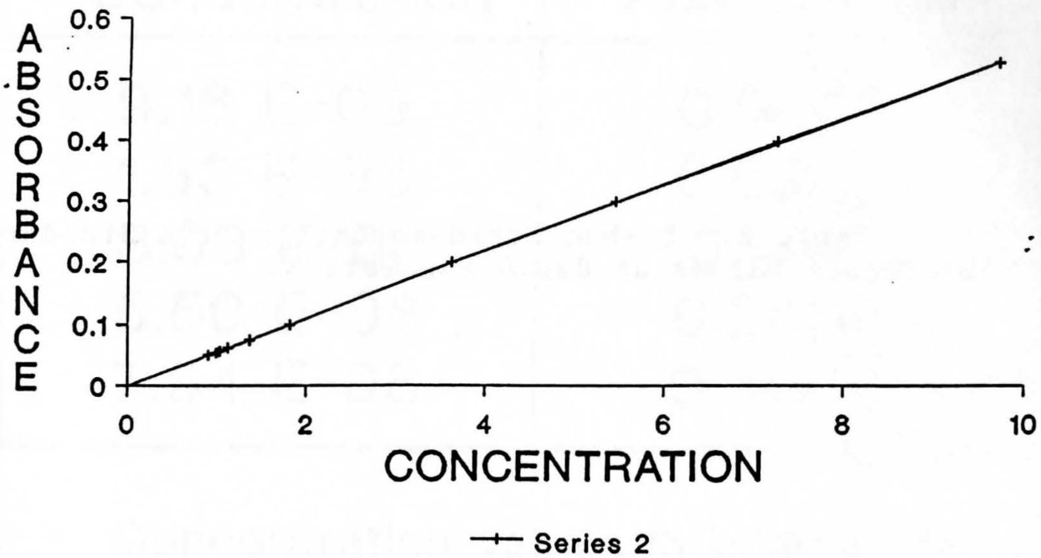
* Ordinate values in units of absorbance.

the 40% sucrose (w/v) layer. The fractions (numbered 3 - 8) are numbered relative to one another with the volume of each dependent on the total number of collected fractions (from each isolation) divided into the total volume of the linear sucrose gradient (approximately 29 milliliters). 5'-nucleotidase activity was highest in these respective fractions indicating that the area from the third to the eighth fraction, presented in relative numbers, is the location of the major plasma membrane band. Fractions listed in Table 1 were determined to be statistically significant at less than the 10 E-06 level when compared to the other fractions regarding the presence of 5'-nucleotidase. The two-tailed Student's t-test was used at a 95% confidence interval. Taking into account the slight variability in the volume of each respective fraction, the major plasma membrane fraction bands out somewhere throughout the 8.75 milliliter range as indicated in Table 3 and Figure 3. Furthermore, since fractions are collected from the bottom up, this would place the most likely area of banding approximately 8.25 milliliters from the bottom of the tube near the middle of the 40% sucrose (w/v) layer, since each gradient layer is 6 milliliters in volume as shown in Figure 3.

Each of the plasma membrane fractions listed in Table 1 was further screened for microsomal, lysosomal, and mitochondrial contamination by enzymatic assays utilizing the marker enzymes glucose-6-phosphatase, acid phosphatase, and cytochrome oxidase, respectively. All fractions were found to

Figure 2. 5'-Nucleotidase Calibration Curve with Plasma Membrane Fraction Values also Plotted.

5'-Nucleotidase Calibration Curve
With Plasma Membrane Fraction Values



Concentration units are 10 E-08 g/ml

Table 2. 5'-Nucleotidase Calibration Standards Absorbance Values at 820.0 Nanometers.

5'-Nucleotidase Calibration Curve: Calibration Standards Data

Concentration *	Absorbance
9.16 E-09	0.0497
1.83 E-08	0.0995
3.66 E-08	0.1990
5.50 E-08	0.2985
7.34 E-08	0.3980

* Concentration values in g/ml units

be free of contamination.

The contaminant-free membrane fractions were then prepared for HPLC analysis. Since the membrane fragments were isolated in sucrose gradients, it was necessary to show that sucrose did not interfere with the analysis of the plasma membrane protein profiles. As previously stated, this was done by running a spectrophotometric scan of a .001 M sucrose from 190.0-300.0 nanometers. The results which are presented in Table 4 and Figure 4 indicated that sucrose absorbs maximally at 209.0 nanometers with a lesser peak at 227.0 nanometers. Since HPLC analysis of membrane glycoproteins was done at 280.0 nanometers, it is evident that sucrose interference with the protein fractions under study did NOT occur therefore eliminating the need for its removal. The maximum absorbance value of 209.0 nanometers has also been verified by a technical representative of the Sigma Chemical Co. from which the sucrose was obtained.

HPLC analysis was then carried out on all the fractions listed in Table 1. The plasma membrane fractions were stored in liquid nitrogen prior to their use. Our studies demonstrated that liquid nitrogen keeps plasma membrane fractions from being degraded and thus preserved enzymatic activity (Yemma and Stroh, 1991). Samples were always removed from liquid nitrogen immediately before HPLC analysis. After preparation of HPLC samples, they were kept at 4° C until actual injection of the sample onto the column. Figures 5 - 6 show the differences between the plasma membrane profiles of

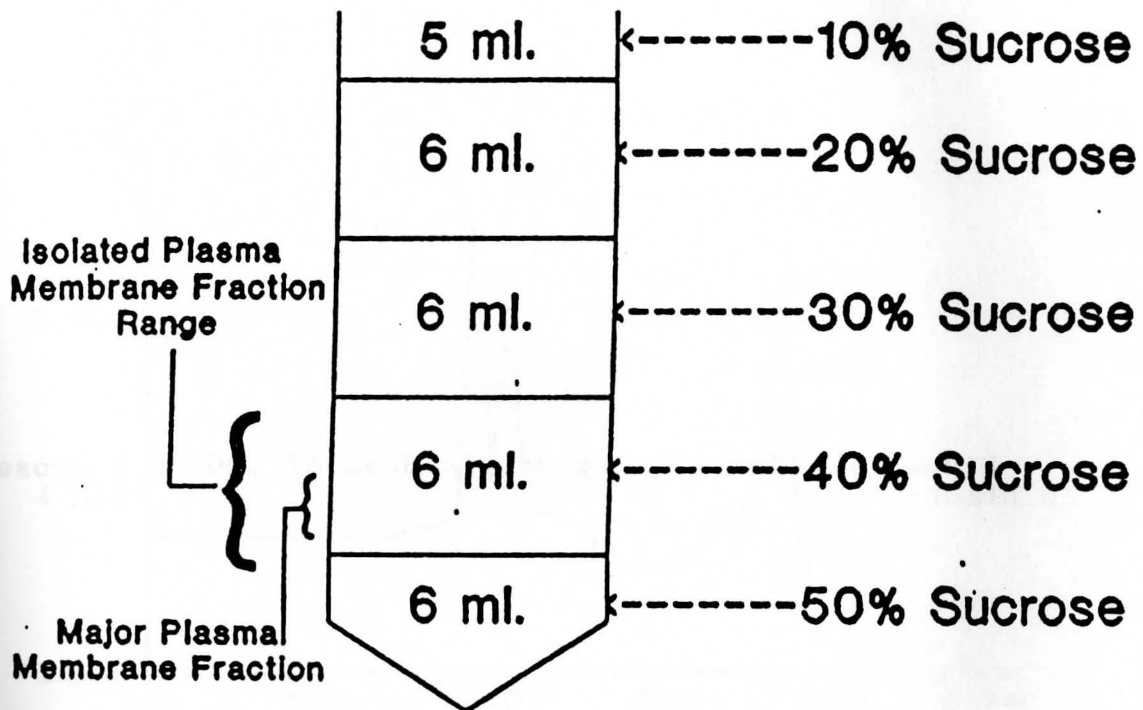
Table 3. Location of Isolated Plasma Membrane Fractions in the Linear Sucrose Gradient.

Plasma Membrane Isolation Results

Collected Sucrose Gradient Aliquots

Sample I. D.	Sample Locations In Gradient (ml.)	Membrane Type
5(a)	8.29 -- 10.35	Induced
5(b)	4.29 -- 5.35	Induced
8(a)	7.50 -- 8.56	Induced
5(c)	8.29 -- 10.35	Uninduced
3	3.63 -- 5.43	Uninduced
4	5.44 -- 7.24	Uninduced
5(d)	7.25 -- 9.05	Uninduced
8(b)	11.28 -- 12.88	Uninduced

Figure 3. Location of Isolated Plasma Membrane Fractions in the Linear Sucrose Gradient.


5 ml.
10% Sucrose
6 ml.
20% Sucrose
Isolated Plasma Membrane Fraction Range
6 ml.
30% Sucrose
6 ml.
40% Sucrose
6 ml.
50% Sucrose
Major Plasma Membrane Fraction

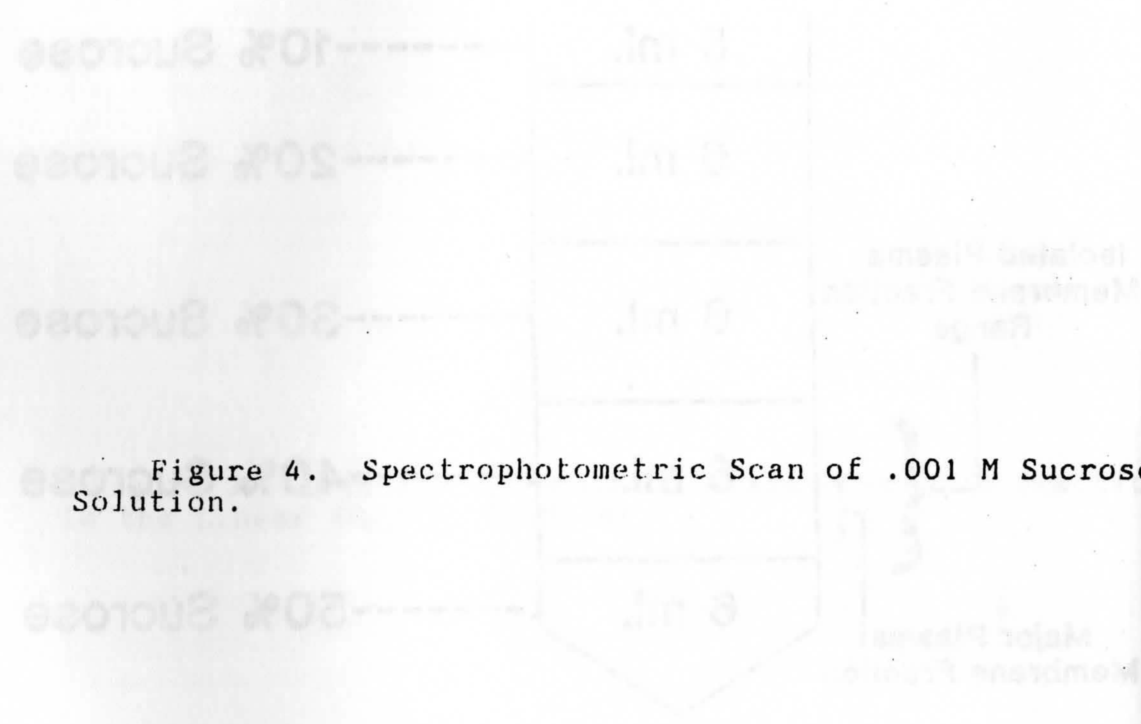
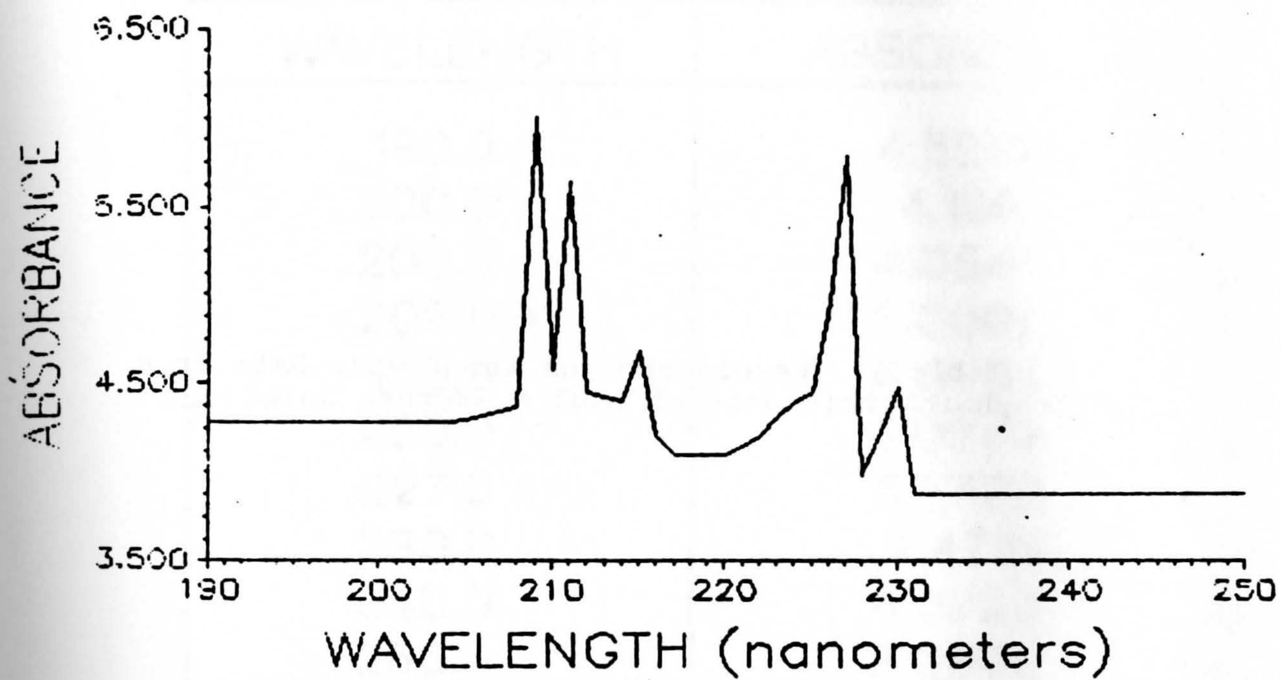


Figure 4. Spectrophotometric Scan of .001 M Sucrose Solution.



6.500
5.500
4.500
3.500
ABSORBANCE
190
200
210
220
230
240
250
WAVELENGTH (nanometers)

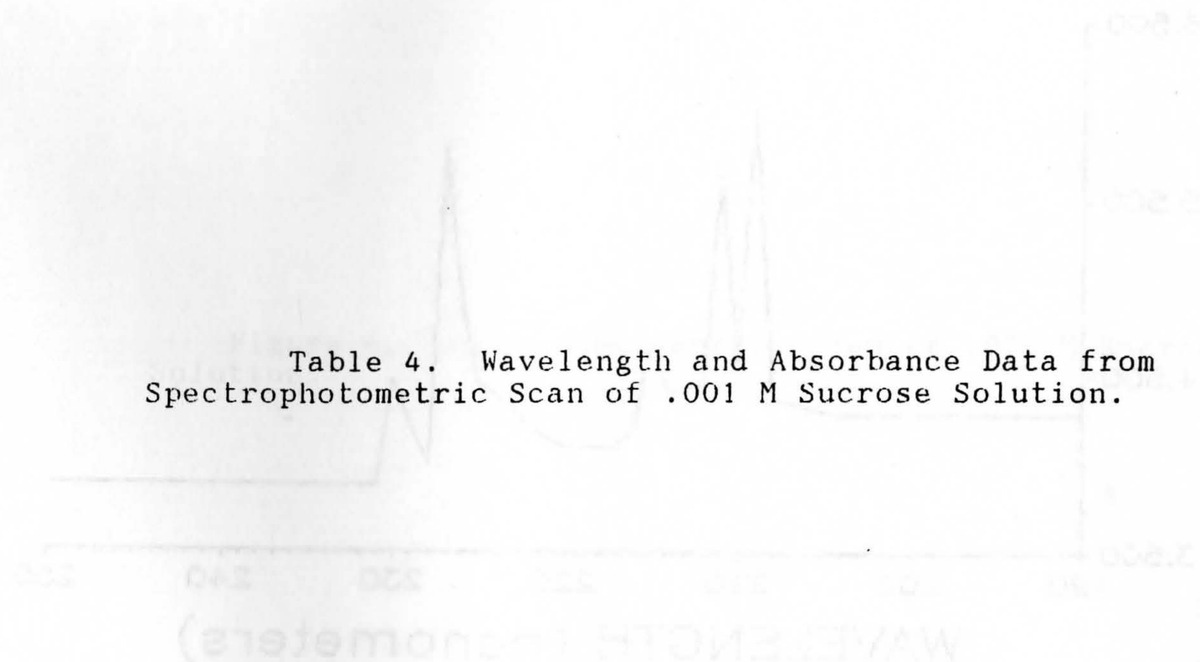


Table 4. Wavelength and Absorbance Data from Spectrophotometric Scan of .001 M Sucrose Solution.

Spectrophotometric Data: Scan of .001 M Sucrose Solution from 190.0--250.0 Nanometers

WAVELENGTH*	ABSORBANCE
190.0	4.5256
200.0	4.1061
208.0	4.0547
209.0 **	6.0000 **
210.0	4.5706
220.0	4.0343
227.0	5.7738
230.0	4.4710
240.0	4.2020
250.0	3.8868

** maximum absorbance

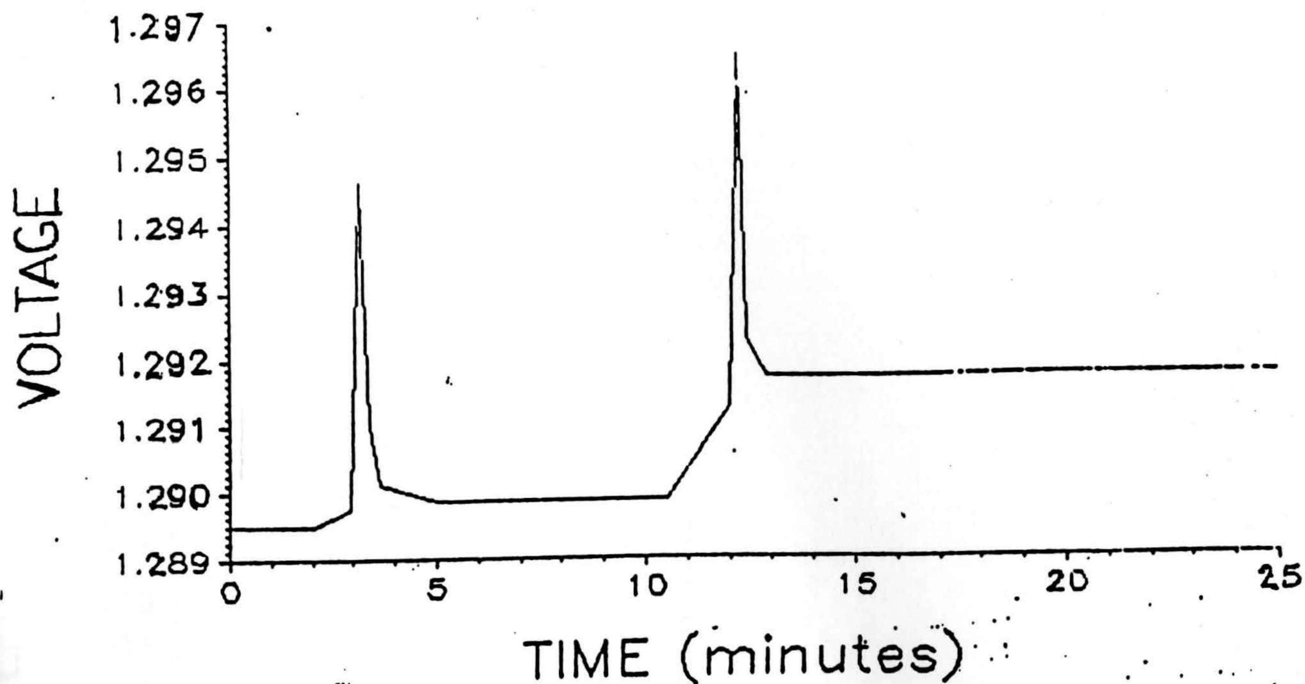
* Wavelength values are in nanometer units

isolated induced and uninduced plasma membranes prepared by HPLC analysis. Comparison of these plots along with the peak data presented in Tables 5 - 6 indicate fundamental differences in constituent protein structure between induced and uninduced plasma membrane protein profiles. All plots exhibited a common peak at approximately 3.12 +/- .09 minutes which varied in height for both induced and uninduced fractions. Since this peak at 3.12 minutes is common in both induced and uninduced fractions, it is of little interest in demonstrating differences between pre-fusion and fusion competent membranes unless it was much more pronounced in induced fractions as compared to uninduced fractions. Comparison of all the plots in Figures 5 - 6 and the data in Tables 5 - 6, demonstrated the area of interest as being between 11 and 15 minutes after sample injection. Taking into account the data from induced plasma membrane HPLC analysis in Table 5, the major plasma membrane protein peak is eluted at approximately 12.06 +/- .27 minutes. A prominent peak in each of the induced plasma membrane fractions is shown here. Data from the uninduced plasma membrane fractions presented in Table 6 with plots exhibited in Figure 6 indicate two prominent peaks, with the first being eluted at approximately 11.20 +/- .09 minutes and the second eluted at 14.83 +/- .09 minutes. The peak at 11.20 minutes is usually more pronounced than the peak at 14.83 minutes.

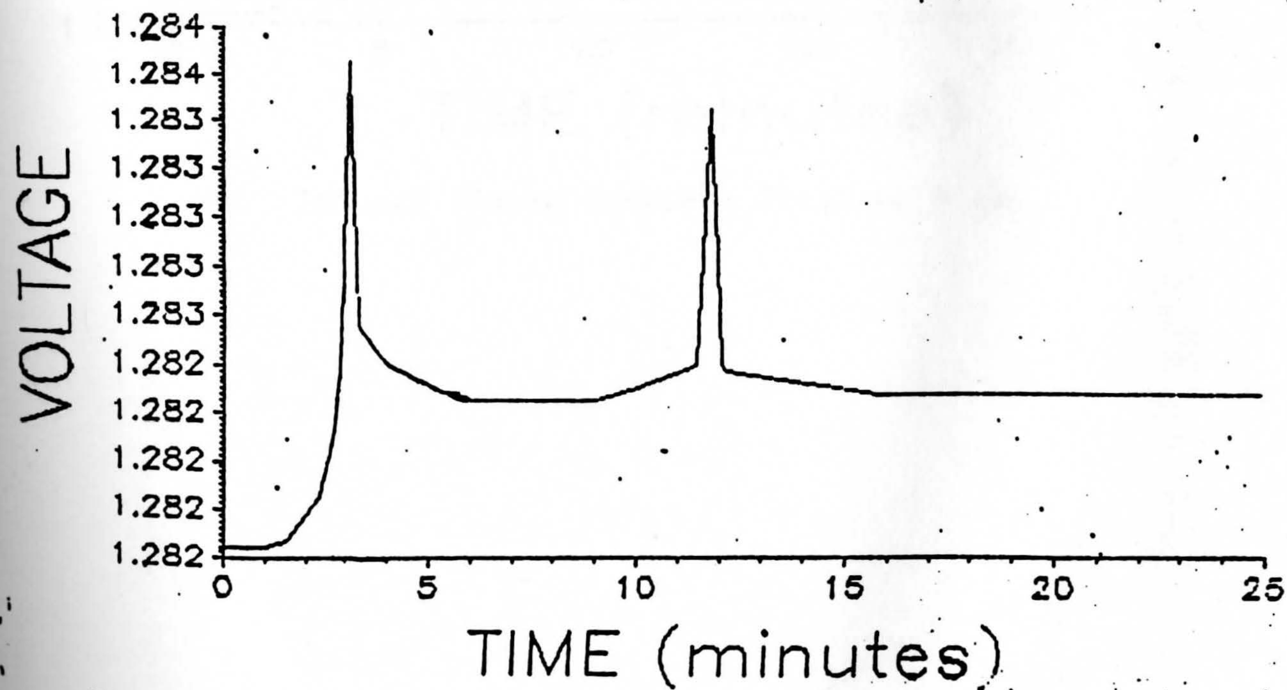
Peak comparisons are significant because differences were shown to occur over more than a single peak. Therefore,

a single membrane surface glycoprotein did not represent differences between the protein profiles of induced and uninduced membranes. Instead, several proteins were shown to be involved in the mechanism of cellular fusion in D. **iridis**. Plasma membrane protein peaks at 11.20 and 14.83 minutes were exhibited in the HPLC analysis of uninduced plasma membranes (Figures 6a - 6e), but induced membrane fractions were characterized only by peak elution at 12.06 minutes (Figures 5a - 5c) over the time period of interest (11 to 15 minutes). Differences in peak elution of membrane proteins in uninduced versus induced plasma membrane fractions as analyzed in Figures 5 - 6, indicated that pre-fusion and fusion competent membranes are differentiated from each other at the biochemical level by more than a single membrane surface glycoprotein (fusion receptor). The significance here thus becomes focused on the induction period prior to membrane fusion as indicated by Yemma and Perry (1985). Cells in an uninduced state exhibited membrane protein profiles characterized by peak elution at 3.12 minutes, 11.20 minutes, and 14.83 minutes (Figures 6a - 6e). Plasma membrane protein profiles of induced cells, though, demonstrated a significant difference in comparison to uninduced cells as two protein peaks were eluted, at 3.12 minutes and 12.06 minutes (Figures 5a - 5c). Results presented here indicate that restructuring through conformational changes involved more than one membrane surface protein as shown in Figures 5 - 6.

Figures 5a - 5b. High Performance Liquid Chromatography Analysis of Isolated Induced Plasma Membrane Fractions of the Myxomycete *Didymium iridis*.

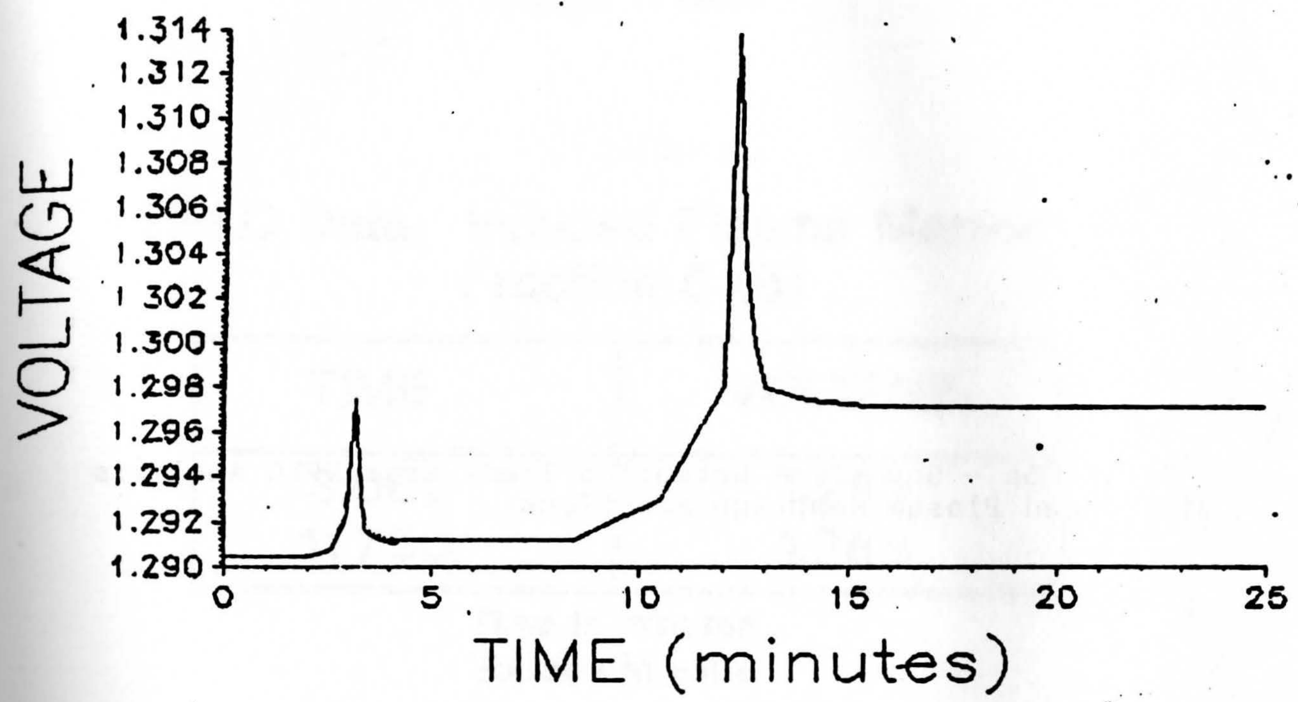


5a. Induced Plasma Membrane Fraction 5 (a)



5b. Induced Plasma Membrane Fraction 5 (b)

Figure 5c. High Performance Liquid Chromatography Analysis of Isolated Induced Plasma Membrane Fractions of the Myxomycete Didymium iridis.



5c. Induced Plasma Membrane Fraction 8 (a)

Tables 5a - 5c. Peak Retention Times from HPLC Analysis of Induced Plasma Membrane Fractions.

Table 5a.

HPLC Data: Induced Plasma Membrane Fraction 5 (a)

TIME	VOLTAGE
3.160	1.294
12.180	1.296

Time in minutes
Voltage in volts

Table 5b.

HPLC Data: Induced Plasma Membrane Fraction 5 (b)

TIME	VOLTAGE
3.060	1.283
11.750	1.283

Time in minutes
Voltage in volts

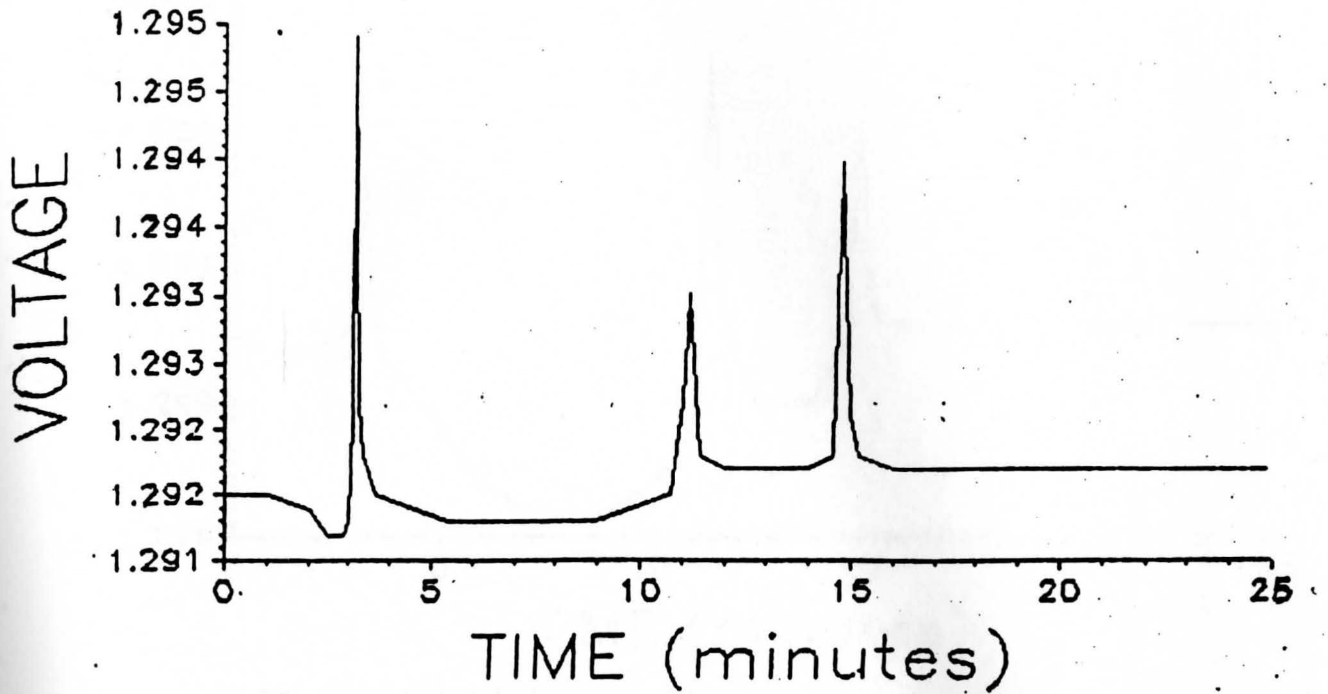
Table 5c.

HPLC Data: Induced Plasma Membrane Fraction 8 (a)

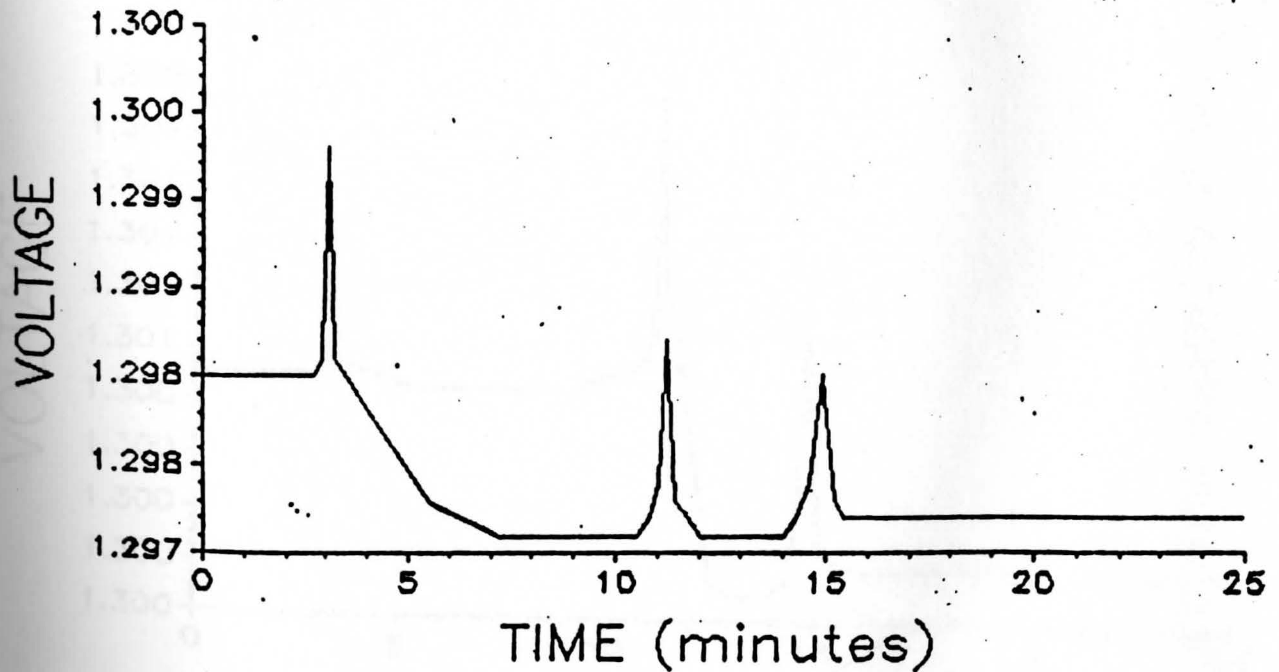
TIME	VOLTAGE
3.180	1.297
12.250	1.313

Time in minutes
Voltage in volts

Figures 6a - 6b. High Performance Liquid Chromatography Analysis of Isolated Uninduced Plasma Membrane Fractions of the Myxomycete **Didymium iridis**.

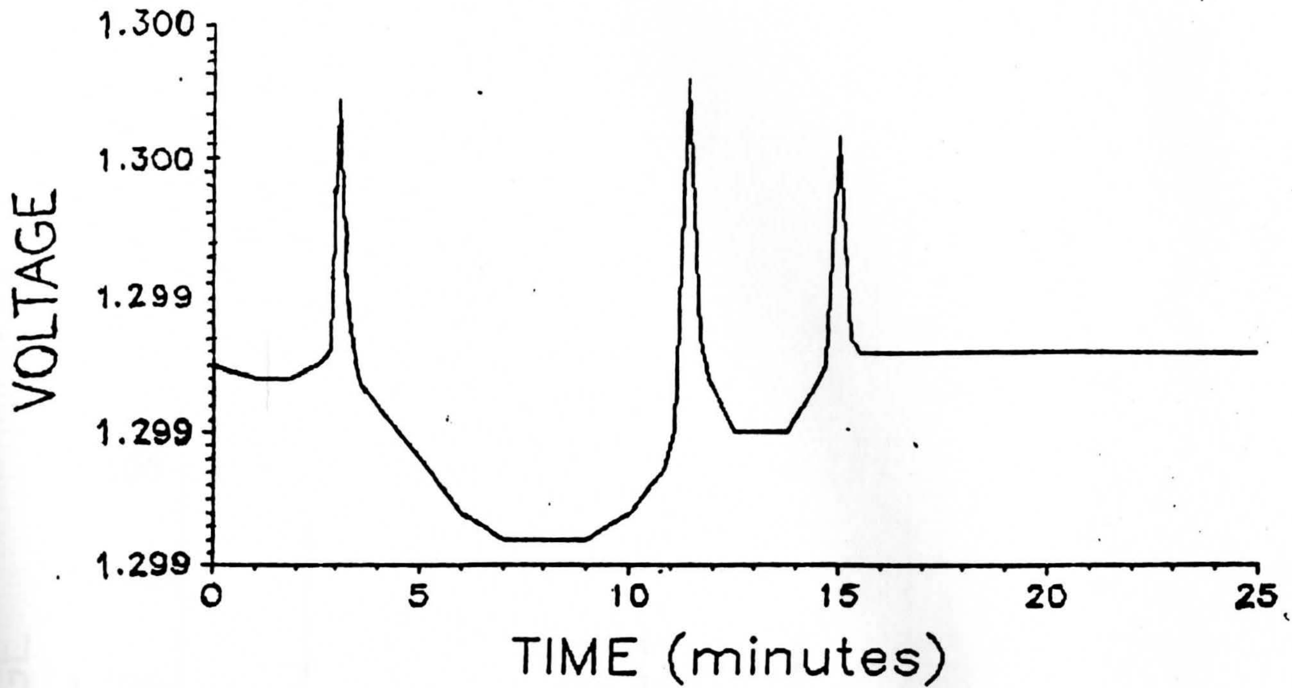


6a. Uninduced Plasma Membrane Fraction 5 (c)

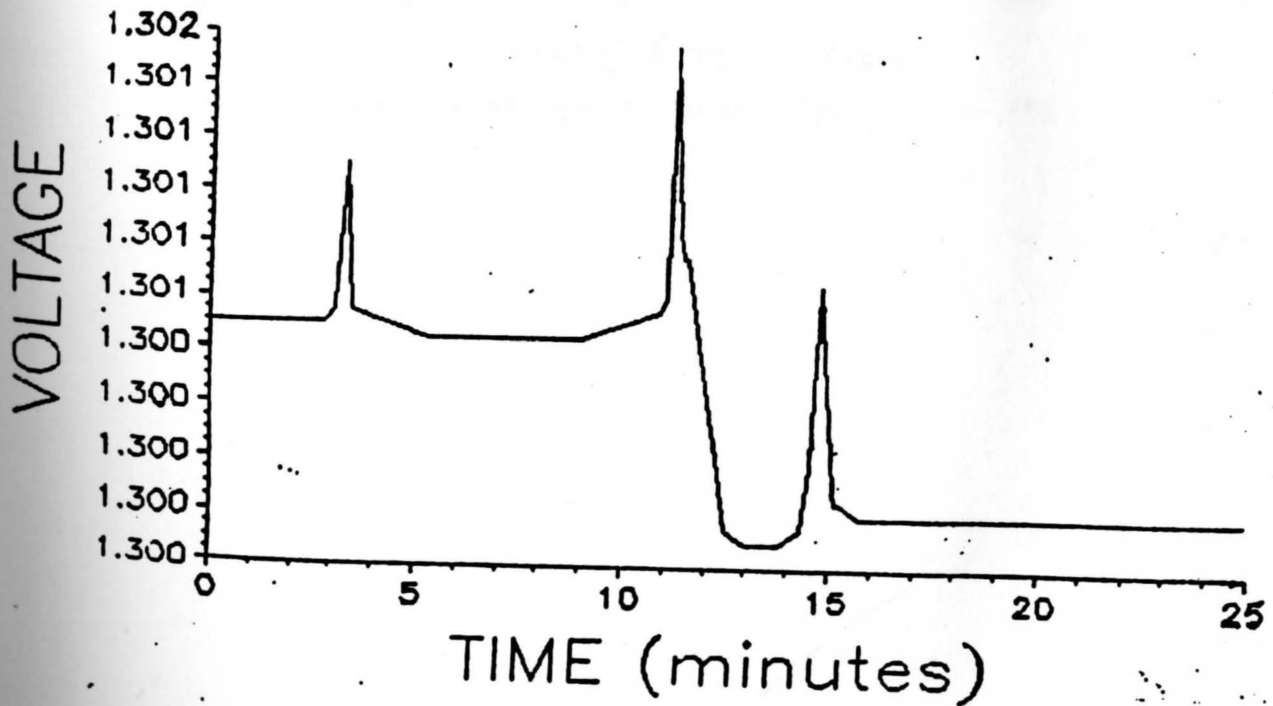


6b. Uninduced Plasma Membrane Fraction 3

Figures 6c - 6d. High Performance Liquid Chromatography Analysis of Isolated Uninduced Plasma Membrane Fractions of the Myxomycete **Didymium iridis**.



6c. Uninduced Plasma Membrane Fraction 4



6d. Uninduced Plasma Membrane Fraction 5 (d)

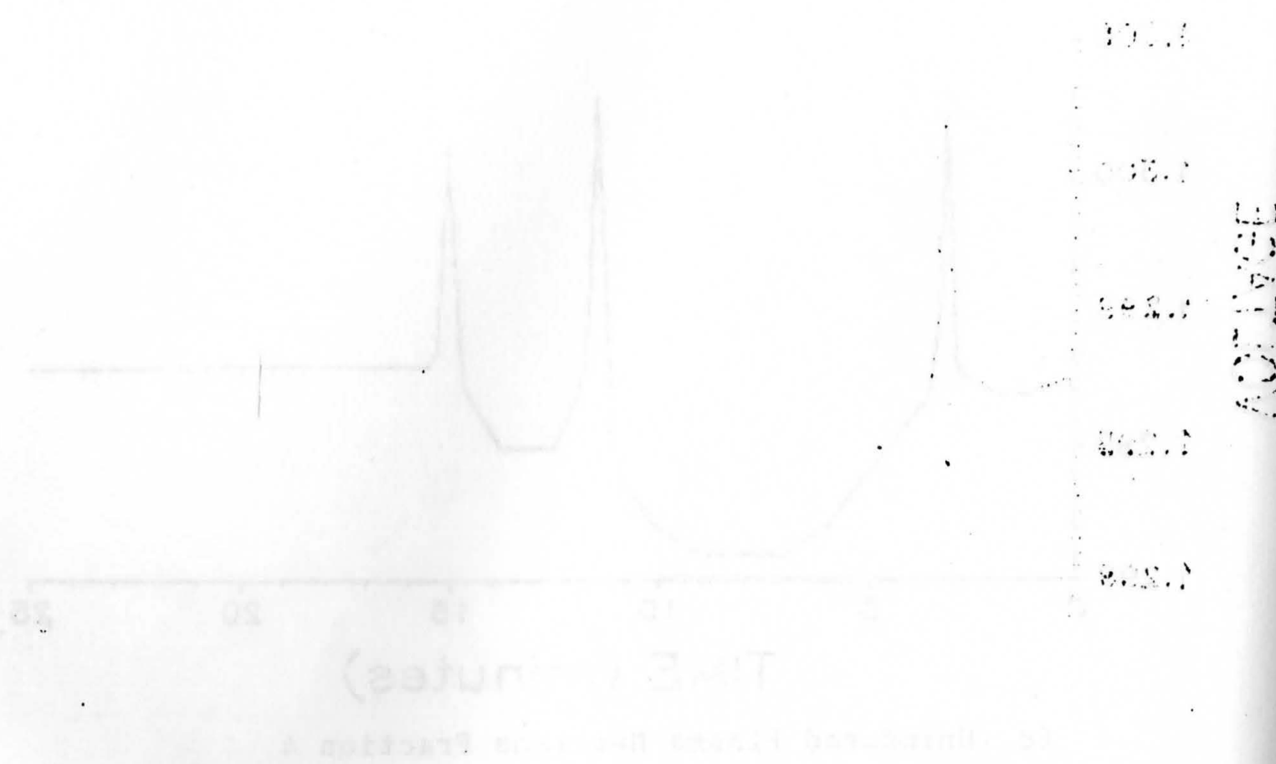
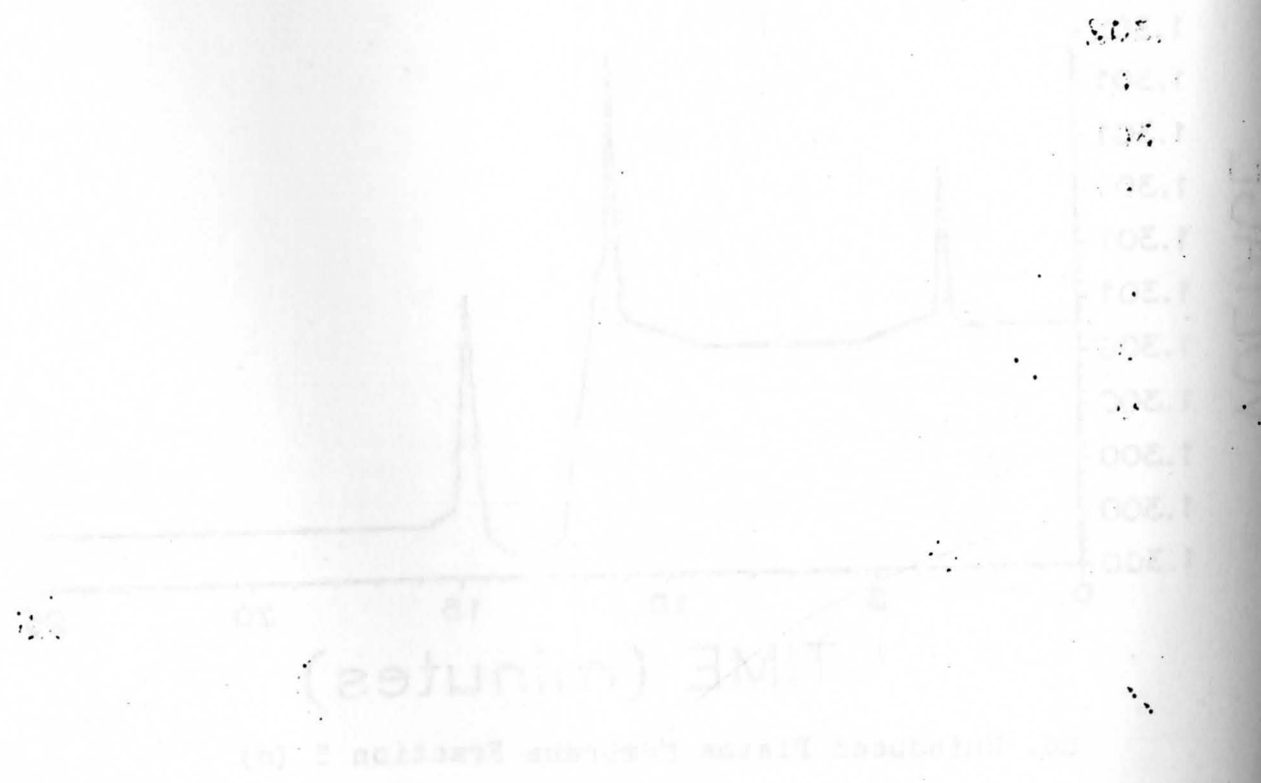
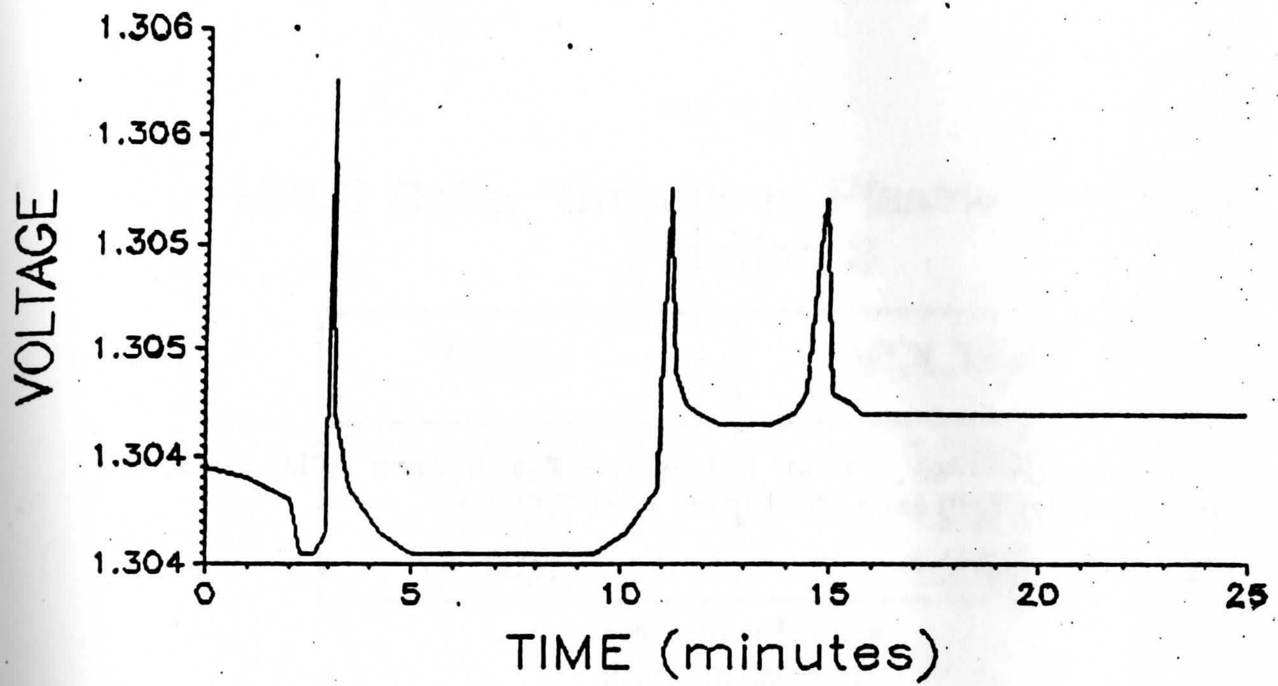


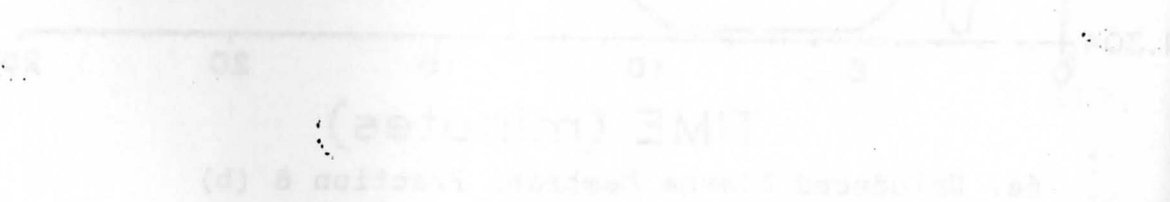
Figure 6e. High Performance Liquid Chromatography Analysis of Isolated Uninduced Plasma Membrane Fractions of the Myxomycete Didymium iridis.





6e. Uninduced Plasma Membrane Fraction 8 (b)

Tables 6a - 6c. Peak Retention Times from HPLC Analysis of Uninduced Plasma Membrane Fractions.



HPLC Data: Uninduced Plasma Membrane Fraction 5 (c)

TIME	VOLTAGE
3.160	1.295
11.160	1.293
14.740	1.294

Time in minutes
Voltage in volts

Table 6b.

HPLC Data: Uninduced Plasma Membrane Fraction 3

TIME	VOLTAGE
3.050	1.299
11.220	1.298
14.900	1.298

Time in minutes
Voltage in volts

Table 6c.

HPLC Data: Uninduced Plasma Membrane Fraction 4

TIME	VOLTAGE
3.050	1.300
11.350	1.300
14.940	1.300

Time in minutes
Voltage in volts

Tables 6d - 6e. Peak Retention Times from HPLC Analysis of Uninduced Plasma Membrane Fractions.

Table 6d.

HPLC Data: Uninduced Plasma Membrane Fraction 5 (d)

TIME	VOLTAGE
3.290	1.301
11.150	1.301
14.770	1.300

Time in minutes
Voltage in volts

Table 6e.

HPLC Data: Uninduced Plasma Membrane Fraction 8 (b)

TIME	VOLTAGE
3.030	1.306
11.120	1.305
14.800	1.305

Time in minutes
Voltage in volts

CHAPTER IV

Discussion

The process whereby cells recognize and adhere to each other and fuse is obviously one of the most crucial and complicated events in biology. In mammalian systems, fusion between cells to form tissues and organ systems occurs over a period of several months or years. In the myxomycetes, these events occur over a period of hours or days. Thus, these organisms provide an ideal system regarding research in developmental biology, in, for example, those studies involving cell receptors and surface adhesion molecules, and their role in cell fusion. There has been a significant number of studies reported in the literature concerning the mechanism of this process largely done using the cellular slime mold (Dictyostelium discoideum in particular) as a model. However, very few attempts if any have been made at characterizing this process in individual cells. Therefore, the present study reasonably focuses on the plasma membrane since these events are centered there. Understandably, it was important to isolate and characterize it regarding proteins or glycoproteins that may be important in membrane fusion in the myxomycete **D. iridis**.

Until the work of Yemma and Selanik in 1976, no attempt had been made at isolating the plasma membrane in the acellular slime mold **D. iridis**. At this time, the majority of the protocols for the isolation of the plasma membrane in

other systems utilized the process of gradient density centrifugation. This method at that time proved unfruitful. Yemma and Selanik (1976) initially attempted this isolation procedure in a sucrose gradient but the result was a low plasma membrane yield which was relatively impure. The band representing isolated plasma membranes in that study was found at the interface between 40%/45% sucrose. The low membrane yield and fraction impurity were attributed to cell fraction damage caused by long centrifugation times at low speeds, and also to the fact that a fixed angle rotor was employed.

In 1983, Barden et al. successfully attempted to isolate and characterize the plasma membrane of another myxomycete, **Physarum polycephalum**. Regarding this study, an initial attempt was made utilizing the two-phase polyethyleneglycol (PEG) polymer system of Yemma and Selanik (1976). This method provided isolated membranes on the basis of molecular shapes and sizes, but it proved problematic in terms of their yield. Since the yield had to be large in the present study, initial attempts at plasma membrane isolation utilizing the two-phase PEG polymer method of Yemma and Selanik (1976) gave an inadequate volume, but pure yields.

Later, incorporating some modifications, Barden et al. (1983) developed a gradient method which proved to be successful using the organism **P. polycephalum**. In an attempt to locate the plasma membrane fraction(s) by enzymatic assay, peak activity was located in the fraction composed of 38% sucrose (w/w) in a linear sucrose gradient.

Using this method with some major modifications, plasma membrane isolation in **D. iridis** was successfully carried out in the present study. Also, it is important to add, one of the goals of this study was to isolate and characterize the plasma membrane surface structure involved in membrane fusion. With this in mind, it was initially necessary to treat **D. iridis** myxamoeba with as few reagents (preferably inert) as possible, while at the same time to produce an acceptable yield of plasma membrane fractions. It was also necessary to eliminate any extraneous substances which would interfere with both enzymatic assay reactions and HPLC analysis. Treatment of the intact myxamoeba with weak salts (.14M NaCl), and mildly basic buffers (1mM $ZnCl_2$, 10mM Tris-HCl, pH 8.0), served as preparatory steps for cell rupture (via sonication). These reagents then were quickly removed by the drawing off of the supernatant layer following centrifugation. This procedure allowed for a non-destructive suspension medium containing sucrose to be utilized. This treatment did not deleteriously affect the plasma membrane structure and provided optimum conditions for the membrane marker assay to be performed. Fortunately, these conditions also proved compatible with HPLC analysis of plasma membrane mating receptors. For example, maximum spectrophotometric absorbance of membrane glycoproteins is 280.0 nm while sucrose absorbs maximally at 209.0 nm. Another benefit is that the plasma membrane isolation procedure could also be completed within several hours, including collection of cell fractions and

running of enzymatic assays, instead of several days, as was the previous case. High-speed centrifugation over relatively short periods of time, in a refrigerated environment (4° C), was of great benefit, as opposed to low-speed centrifugations over longer periods of time, which could possibly result in deterioration and damage to the cell fractions. The method of Yemma and Selanik (1976), although possibly more rapid in terms of isolation time, permitted the use of low-speed centrifugation when an ultracentrifuge was not available, or its use not desirable. The requirement for removal of phosphate as well as PEG from the phosphate-containing isolation medium in which the membranes were isolated, proved to be problematic when using the two-phase polymer system. This understandably would of course have to be done prior to enzymatic assay where the phosphate ion concentration is measured. The method employed in this work did not present this problem.

Centrifugation in a fixed angle rotor was employed in this study. Moore (1969) reported that such a rotor affected the migration of cellular fragments in a linear sucrose gradient, when compared to centrifugation in a swinging bucket rotor. The results of this study, however, did not support this contention. No adverse affects were exhibited concerning the migration of cellular fragments when centrifugation was carried out in a type 42.1 fixed angle rotor and a Beckman L7 ultracentrifuge. This was verified by the fact that the five successive and separate plasma membrane isolations carried out

in order to test for zone appearance and repeatability provided highly repeatable results. The major area of banding for the isolated plasma membrane fragments as shown by 5'-nucleotidase activity, within the linear sucrose 40% (w/v) gradient was located at about 8.50 milliliters from the bottom of the gradient. These results compared favorably with those of Barden et al. (1983) in which the major band of isolated plasma membranes from **P. polycephalum** was located approximately 7.5 milliliters from the bottom of the isolation gradient in 38% (w/w) sucrose. Isolated plasma membrane fractions were found within a 6-milliliter range of the 40% sucrose (w/v) gradient layer. Any variation in banding was attributed to the initial cell isolation density. Variability in the size of the membrane fragments loaded onto the sucrose gradient (which may have been caused by minor fluctuations in sonication times), and whether or not the membrane fragments are in sheets or vesiculated (Damsky et al., 1984; Maddy, 1984; Gennis, 1989) would account for this. The mean position of the large sample particles in the gradient may be called the equilibrium density, because the particles themselves are in a kind of secular equilibrium (Price, 1982). This study provided evidence to support these findings.

The particles of a homogeneous particle population usually range on either side of their discrete equilibrium density value because of diffusion (Price, 1982; Gennis, 1989). This equilibrium distribution represents an equilibrium between sedimentation (or flotation) which tends

to concentrate at their equilibrium density and diffusion which tends to move the particles away from their equilibrium density. This qualitative model indicates that the width of the isolated plasma membrane particle zone at equilibrium is a function of the centrifugal field, steepness of the gradient, and particle size. It has also been shown that for preformed gradients whose shape is not affected by the centrifugal field, particle zone width is decreased and therefore resolution enhanced by high speeds (Price, 1982). This was verified in this study. Centrifugation in a Beckman L7 type 42.1 rotor at 26,000 R.P.M. for 2 hours, produced a much condensed range over which plasma membrane fractions were isolated, relative to that produced during centrifugation in a fixed angle rotor of an IEC BA-20 centrifuge at 19,000 R.P.M. for 3 hours. In preparative isopycnic separations, particle zone width and therefore resolution is significant with respect to its position in the gradient volume.

Sucrose is commonly used as a solute in homogenization media since it is inert, does not depress the activities of most enzymes, and cellular membranes are relatively impermeable to it (Robinson, 1975). These results were verified in this study. Properties of an ideal solute for aqueous gradients are as follows: freely soluble in water, very dense, nonviscous, negligible osmotic pressure, physiologically and chemically inactive, transparent in UV and visible light, and inexpensive. Sucrose was the gradient of choice for this isolation. Although it is the most widely

used. quite expectedly, it is not completely ideal as a solute for gradients. Of the above listed properties of sucrose, solubility, transparency, and cost are most beneficial. However, physiologically it contains some activity which can prove detrimental to some membranes when isolation is attempted. The results of this study did not demonstrate any detrimental activity to the membranes due to sucrose.

As previously stated, the technique of density gradient centrifugation used in this study is a hybrid of isopycnic and rate zonal separation. This technique depends on the separation of particles on the basis of differences in their buoyant density and on the basis of their sedimentation rate (Gennis, 1989). Often membrane purification protocols are empirically optimized and represent a hybrid separation due to sedimentation coefficient differences, and density differences. In an isopycnic separation, sedimentation rate during centrifugation is a function of the size and density of a particle as well as of the imposed centrifugal force (Robinson, 1975). This technique also allows for the execution of isopycnic centrifugation in which the particles sediment out at a point in the gradient at which the density of the solution is equal to the density of the particle and the sedimentation velocity is therefore zero (Price, 1982). This proved to be an important consideration in the present work, for membrane preparation in the studied cell line is difficult to achieve, as the cells are no more than 4 microns

in diameter, and often less. Due to this characteristic, it was necessary that large volumes of cells be harvested. Also, membranes of various organelles sediment very near one another regarding the respective densities of each. The density of the medium at which a class of organelles equilibrates is called the "buoyant density." In order to separate different membrane fractions from a cellular homogenate, it is often necessary to take advantage of these differences in density. This can be done by centrifugation in a density gradient of a well chosen centrifugation medium (Gennis, 1989). In this case it proved to be sucrose. For separation of membrane fractions, sucrose concentrations must be high enough to be hypertonic, yet still must retain a high viscosity. Sucrose serves to stabilize the membrane suspension in the centrifuge tube, against convective disturbances (which may be caused by high speed spins in a fixed angle rotor) (Moore, 1969). Although centrifugation at 26,000 R.P.M. in a type 42.1 fixed angle rotor, a Beckman L7 ultracentrifuge produced no such deleterious results. Also, it is important to note that buoyant density is in no way an absolute value as it is influenced by the medium and depends on whether the membranes form sheets or enclosed vesicles during homogenization. The literature suggests that because of the high osmotic potential of sucrose, membrane particles might be permeated by the medium causing the particles to form enclosed vesicles. This would alter the density of those affected particles, causing them to band out at a different location in a gradient. The

results of this study demonstrated that the technique used in the isolation of membrane particles resulted ideally in the isolation of membrane fragments in the form of sheets and not in vesicles. Isolation of the external plasma membrane often requires the use of mild shearing conditions so as to prevent undue disruption of membranes, and often facilitates the isolation of resultant larger membrane fragments. Vigorous shearing to produce small membrane fragments, which under some circumstances may be more easily separated from contaminants is also often employed (Robinson, 1975). Mild shearing conditions used in this study, provided by sonication periods for one and five seconds, respectively, resulted in good cell rupturing, and facilitated the isolation of larger desirable membrane fragments.

Price reported in 1982 that isopycnic separations are characterized by the migration of cellular particles into the gradient at a progressively slower rate until they reach a level in the gradient where the densities of particles and medium are the same. Therefore, the final equilibrium position of the particle will be independent of the route, except in the case of particles sensitive to high concentrations of the gradient solute. The use of sucrose as a medium for isolation did not appear to affect the isolation in this study as attested to by the reproducibility of the results in each separate isolation. If sucrose had affected the migration of cellular particles by altering their respective densities, the banding position in the sucrose

gradient of isolated plasma membranes between separate isolation runs would have been different. This was not found to be the case; each successive and separate membrane isolation produced a major area of banding for isolated plasma membranes at 1.17 grams/milliliter. These results are given in Table 3 and Figure 3 of the Results chapter. It was crucial to this study that sucrose did not interfere with the isolation technique, because in addition to the fact that it was cost-effective and easy to work with, it provided the repeatability necessary to make this isolation technique effective while not necessitating its removal prior to HPLC analysis, because of a spectrophotometric absorbance different from that of the membrane glycoproteins under study.

The particular combination of methods and conditions used in isolating any one membrane will depend upon the nature of the membrane and upon the kind of tissue or cell wherein the membrane is located (Robinson, 1975; Gennis, 1989). Even in the case of cultured cells when only one species is present, particular membranes may be derived from cells in different stages of division and so differ in their properties (Price, 1982). Membrane composition varies widely from organism to organism, tissue to tissue, and organelle to organelle. Furthermore, the composition may alter with age or with environment and results of analyses are influenced by the purity of preparations and by the presence of adsorbed contaminants enclosed in the membrane vesicles formed during homogenization (Price, 1982). As indicated by the results of

this study, the major isolated plasma membrane fragments were found to be nonvesiculated. In view of this, it was advantageous to harvest cells initially plated on the same date and time, for isolation to minimalize this problem. Early isolation techniques first devised by de Duve and co-workers; Wattiaux-de Connick and co-workers and Beaufay and co-workers using rat hepatocytes, formed the primary method, which was stepwise modified for the plasma membrane isolation technique developed in this study. One of the underlying considerations, in addition to those already mentioned, was to provide for a method which would easily allow for enzyme assay. A large number of enzymes can be monitored, for enzyme groups can be observed to move together at a given rate and to stabilize at definite isopycnic points, thus establishing a class of organelle defined by a density, sedimentation coefficient, and enzyme profile. This information was particularly important in this study because of the necessity of plasma membrane location by enzymatic assay. Thus, the necessity for enzymatic analysis of isolated membrane fractions in order to monitor microsomal or mitochondrial contamination was of the utmost importance. Until now, adaptation of this technique had never been attempted regarding the myxomycete **D. iridis**. The structural fate of the membrane and the crude fraction in which it will be concentrated it appears is dependent on the method of cell rupture, the composition of the media, and the cell type in question.

Large ghosts, sheets, and brush borders have isopycnic points in sucrose in the range of 1.185-1.16 grams/milliliter (Neville, 1976). If the membranes are in the form of sheets, ghosts, or brush borders, a low gravity pellet enriched in membranes is applied to the density gradient. The application pellets are usually enriched in one or more of the major intercellular organelles and any organelle unique to a particular cell type; this problem affected our isolation also. Of paramount importance, one of the goals of this study was to produce a pure membrane fraction. For as previously stated, enzyme assays provide the necessary validation of the presented method. The degree of enrichment depends on the homogenizing conditions used; shear, ionic strength, pH, concentration of divalent cations, and the gravitational force integrated over time for the application pellet and the conditions of any prior centrifugations (Neville, 1976). Plasma membrane fragments usually sediment out in a gradient at a density between 1.1 and 1.2 grams/milliliter (Gennis, 1989), with some variability attributed to membrane particle size and whether or not the particles are vesiculated or in sheets. As previously mentioned, this study did not exhibit isolation of any vesiculated membrane fragments. Barden et al. (1983) utilized a weight/weight (w/w) percent sucrose gradient, while this study utilized a weight/volume (w/v) percent sucrose gradient, thus accounting for the differences in relative density between equal percentage solutions from the two. Robinson (1975) indicated that in sucrose gradients,

large membrane ghosts, sheets, and brush borders have isopycnic sedimentation points in the range of 1.185-1.16 grams/milliliter while vesicular membrane fractions band between 1.16 and 1.08 grams/milliliter. The sucrose density gradient used in this study and the one utilized in the study of Barden et al. (1983) were both continuous linear gradients from 20% to 50% sucrose, with the former ranging in density from 1.08-1.19 grams/milliliter, and the latter ranging from 1.08-1.24 grams/milliliter. These density values are for sucrose gradients kept at 4° C (Price, 1982), which is the desired temperature for this isolation. Barden et al. (1983), found their major plasma membrane fractions in sucrose at a mean density of 1.16 grams/milliliter. This would indicate that the major isolated plasma membrane in their study did not contain vesiculated particles. In this study, the major area of isolated plasma membrane sedimentation was near the middle of the 40% (w/v) sucrose layer between 44%-45% (w/v) sucrose. Sucrose at a weight/volume percentage of 44.53% is equivalent to a density of 1.172 grams/milliliter (Price, 1982) thus indicating that the isolated plasma membrane fragments comprising the major band of isolated plasma membranes were not contained in vesicles but were rather in the form of sheets.

As mentioned earlier in this chapter, another goal of this study was to provide evidence indicating differences in the plasma membrane protein profiles of uninduced and induced cells. Yemma and Stroh (1991) demonstrated physiological

differences in these cells. Therefore, a reliable membrane isolation technique is imperative in order to characterize a membrane fusion receptor. There is also a strong need for reliable chromatographic systems to separate glycolipids and glycoproteins such as receptors or other proteins containing membrane spanning regions, which was implicit in that study. Such proteins typically contain sequences of hydrophobic residues and as a result are often insoluble in aqueous solvents and prone to aggregation. Therefore, addition of organic solvents has resulted in an improvement in the solubility of membrane proteins important in HPLC analysis. The organic solvent choice for chromatographic analysis in this study was acetonitrile. Accompanied with .02 M phosphate buffer (pH 6.0), the effectiveness of the mobile phase and membrane preparation in glycoprotein separations is supported by preliminary studies done in our laboratory (Yemma and Green, 1988). Moreover, the presence of a detergent is not required, since the organic solvent used for elution prevents aggregation and keeps the membrane proteins in solution. Therefore, the use of a detergent was not employed to treat the cells used in this study.

The chosen method for the preliminary isolation of these plasma membrane proteins was by reversed phase high performance liquid chromatography (RPHPLC). The principle of reversed phase chromatography (RPC) is centered around the fact that as a protein traverses the column, it unfolds to a greater or lesser extent and its interior segments, which are

usually rich in hydrophobic residues, become exposed. This results in their interaction with the chromatographic surface. RPC is so-called because it utilizes a nonpolar stationary phase and a polar mobile phase. The pore size of the stationary support also has a large effect on the efficiency of the separation. The pore size of the Aquapore RP-300 column was 7 microns. Column efficiency is dependent on particle size and size distribution as well as pore size and structure and surface properties of the stationary phase (Ahuja, 1989). As a result, the reversed phase system exhibits selectivities different from those obtained by other chromatographic or electrophoretic means. Therefore, the denaturing conditions inherent to RPC have become advantageous in the analytical HPLC of proteins (Frenz et al., 1990). This method of membrane protein separation was chosen because the macromolecules of particular interest are those which serve as receptors or cell fusion factors in **D. iridis** myxamoeba and which have been postulated as being glycoprotein in nature (Yemma and Soltis, 1988). The actual retention time of a protein depends on the reversed phase, the organic solvent, and the nature of the mobile phase (Ahuja, 1989). When reversed phase chromatography is applied to glycoproteins possessing multiple oligosaccharide attachment sites, elution of these glycoproteins occurs within a relatively short range despite the heterogeneities of the carbohydrate chains (Takahashi and Putnam, 1990). This indicates the relatively minimal effect carbohydrates exert on chromatographic

analysis. However, the heterogeneities of the carbohydrate chains results in an asymmetrical peak containing multiple components. Chromatographic analysis of both induced and uninduced membrane fractions in this study exhibited separation of several membrane glycoproteins. The major glycoprotein peaks focused on in this thesis and exhibited in Figures 5-6 did reflect some asymmetry, possibly due to this fact. Furthermore, these proteins undergo conformational changes both in the mobile phase and at the surface of the stationary phase. Conformational changes and denaturation due to unfolding can complicate the retention process in RPC of proteins, (Frenz et al., 1990). The behavior of macromolecular eluites usually mandates the use of gradient elution for the separation of protein mixtures (Frenz et al., 1990). This proved to be the case in this study using a linear gradient, including acetonitrile and .02 M phosphate buffer (pH 6.0).

When employing RPC with regard to protein separation, gradient elution is most commonly used, and thus the organic solvent content of the eluent is increased gradually during the chromatographic run. In this study, however, the organic solvent was gradually decreased throughout the run by being combined with .02 M phosphate buffer (pH 6.0), until the resulting eluent was in a 50:50 mixture at the end of the run. The solvent gradient reduces the surface tension of the water molecules thus decreasing the energy required to form a cavity of water molecules surrounding nonpolar molecules. With a

sufficient decrease in surface tension, the sample molecule is no longer adsorbed to the reversed phase and is eluted in the mobile phase. In RPC of proteins, the balance of eluting and denaturing properties of the mobile phase is also an important consideration. The nature of the buffering system (.02 M phosphate buffer, pH 6.0), the organic solvent (acetonitrile), and other additives all contribute to these functions.

The column efficiency of protein separations is flow rate-sensitive and better results were obtained when relatively low flow rates were used. In this study, samples were run at flow rates ranging from .5-2.0 milliliters/minute with the optimal flow rate determined to be at 1.0 milliliter/minute. Aside from its effect on column dynamics, the flow rate has been shown to influence the conformational integrity of a protein in HPLC by affecting the time the molecule is adsorbed on the chromatographic surface (Frenz et al., 1990). Relatively slow conformational changes of the protein become manifest at low flow rates since longer contact between the eluite and stationary phase allows more time for unfolding of the protein. It was noted that column temperature also had an effect on the efficiency of the separation and conformational status of the protein during the separation, but since the column was kept at a constant temperature of 25° C by use of an oven, only slight variation could be attributed to temperature effects.

Clearly the tertiary structure and conformational instability of proteins introduces additional complications to

RPC. Since the composition of the eluent changes during the chromatographic run, the mobile phase conditions from which the protein is loaded onto the column may differ substantially from those in which it is eluted which may produce conformational changes including aggregation or disaggregation of the protein. Therefore, the complexity of the chromatographic behavior of proteins necessitates the proper selection of the mobile and stationary phases. The other operating parameters must also be chosen with care so as to achieve the appropriate binding strength and conformation state for optimal resolution and column efficiency. This serves to emphasize the importance of a pure membrane preparation. The use of wider pore supports is preferable due to the high molecular weight of proteins and a preference for wider pore silicas such as Aquapore RP-300, which is the column of choice in this study. When stationary phases are of moderate hydrophobicity, they can alleviate the severe denaturation and sample loss that can be observed with other reversed phase columns.

The initial hypothesis of this study regarding membrane fusion focused on the existence of a single receptor site at which induced myxamoeba could come into physical contact with one another, fuse and form diploid zygotes. However, Yemma and Stroh (1991) mentioned the possibility of mating factors. Evidence regarding the chemical characterization of this receptor or factor was provided by Yemma and Soltis (1988) in their study indicating blockage of cell fusion using the

lectin concanavalin A, which binds the alpha-mannopyranase and alpha-glucopyranase moieties of glycoproteins. Yemma and Stroh (1991) also indicated that in order for membrane fusion to occur between myxamoeba, these cells must become competent to do so through induction. This can occur by attainment of a critical cell density (1 x 10 E+05 cells/milliliter), by self-induction, or from cross-induction from an already induced cell population. Isolating plasma membrane fragments from both induced and uninduced cells was thus of paramount importance. It was the intention of this study to explore any difference in the plasma membrane protein profiles of induced versus uninduced cells and to demonstrate, if present, a fusion receptor. Evidence illustrating differences in the plasma membrane regarding pre-fusion versus fusion competency, or uninduced versus induced cells, is crucial since it is the induced population that demonstrates fusion competency and, therefore, the existence of mating factors or receptors (Yemma and Stroh, 1991). Theoretically, the presence of a plasma membrane fusion receptor in induced cells, and its absence on uninduced cells, should produce HPLC results in which induced plasma membrane fractions exhibit differences in regard to surface proteins. This change would largely be manifest in conformational changes. It was further hypothesized prior to collecting data for this work that a unique receptor was involved. The fact that uninduced membrane fractions produced two protein peaks while induced membrane fractions produced one peak upon HPLC analysis indicates that the surface of the

plasma membrane undergoes changes involving several proteins, after induction, prior to cell fusion. Most reproducibility was shown in HPLC analysis between uninduced membrane fractions from successive and separate plasma membrane isolations and likewise between induced membrane fractions also from successive and separate isolations. The exhibited reproducibility allowed for effective comparison of HPLC results.

The evidence provided indicates that a difference exists between the plasma membrane surface of induced and uninduced cells. HPLC analysis of both induced and uninduced membrane fractions resulted in the elution of several protein peaks. A peak eluted at 3.12 +/- .09 minutes was present in both induced and uninduced fractions. Since the membrane protein represented by this peak is conserved in both pre-fusion and fusion competent cells, it is not likely that it is involved in the membrane restructuring that occurs prior to fusion. The proteins which are involved in the restructuring of the membrane surface prior to fusion are eluted between 11 and 15 minutes. Results presented in Figures 5 - 6 illustrate the elution of two protein peaks for uninduced membrane fractions, at 11.20 and 14.83 minutes, respectively, while induced membrane fractions produced only one peak at 12.06 minutes over the time period of interest. Because the induction period prior to fusion is short, membrane restructuring must occur quickly. Therefore, the membrane protein eluted in induced membrane fractions cannot be

produced de novo. It must instead arise from a restructuring of the membrane surface through conformational changes of proteins already present on the plasma membranes of uninduced cells. The protein peak eluted at 12.06 minutes in induced membrane fractions appears to represent the glycoprotein plasma membrane fusion factor. The conformational changes which the proteins undergo on the membrane surface most likely result in the expression of this membrane glycoprotein fusion factor at 12.06 minutes at the induced membrane surface along with the repression of the two proteins present on the uninduced membrane surface at 11.20 and 14.83 minutes, respectively. It is also possible that this protein eluted by induced membrane fractions represents a hybridization of the two membrane surface proteins eluted by uninduced membrane fractions. The two proteins eluted by uninduced membrane fractions may act as precursors in the formation of a single glycoprotein membrane fusion factor. In light of the data presented, it seems clear that although some light has been shed on the phenomenon of cell fusion, which includes this work as well as the work of Yemma and Perry, 1985; Yemma and Soltis, 1988; and Yemma and Stroh, 1991, much remains to be done.

It will be necessary not only to continue to focus on the physiology of the process itself, but also to identify the specific participating membrane components. Furthermore, it will be necessary to identify and characterize the glycoproteins present at different developmental stages, and

then subsequently to identify the underlying regulatory mechanisms. Regarding **D. iridis**, evidence has been previously presented by Yemma and Stroh (1991), supporting the presence of an inducer molecule, which is produced by cells once they attain a critical density, and these cells are then capable of fusing when in contact. In these studies, differences had been shown between induced and non-induced cell lines indicating changes brought by the addition of inducer molecules in the uninduced cells.

The information for establishing and maintaining correct cellular contacts must lie in the expression and organization of molecules on the cell surface. This is the principle upon which this study is based. Characterization of a plasma membrane fusion receptor in **D. iridis** can lead to the elucidation of the mechanism of fusion in this organism. Two general approaches have been taken in the past in an attempt to identify the molecules involved in cell-cell adhesion with one of these approaches involving the identification of those surface molecules involved in the aggregation of individual amoeba through use of an antiserum capable of disrupting cell-cell interaction, followed by the elucidation of the identity of those antigens involved. It is interesting to recall that the principal antigen recognized by an antibody may not be the ligand directly involved in holding two cells together, but may instead represent another cell surface molecule somehow required to maintain the structure or organization of molecules at the site of adhesion (Damsky et al., 1984).

Although these molecules are not directly involved at the site of attachment, their presence and proper functioning is required for adhesion to occur, and once these adhesion-related membrane glycoproteins have been identified, their function and the nature of their interactions with one another as well as with components of the matrix and cytoskeleton must be elucidated. Recently, antibodies have been made in our laboratory in rabbits to the HON 1-7 A^2 mating strain of D. iridis. Experiments to be presented in a later study demonstrate that induced and uninduced cells separately incubated with these antibodies and then tagged with fluorescinated goat antibodies show exciting differences. Visual examination of these tagged cells exhibited greater fluorescence on induced cells in comparison to uninduced cells. Fluorescence tagging occurred over the entire surface of the cell indicating the presence of more than a single fusion site in D. iridis.

Extrapolating this information to the mechanism of fusion in D. iridis, though, Yemma and Perry (1985) provided evidence against zygote formation via the mechanism of rapid agglutination of cells. Evidence presented indicated that zygote formation did not occur immediately after mixing of compatible mating types but was in fact time-dependent. It was therefore discovered that a period of induction was necessary to prepare the myxamoeba for fusion (Yemma and Perry, 1985). Earlier, Ross et al. (1973) had suggested that an induction period was required for receptor site synthesis

by postulating a model system concerning the functional aspects of the mating type alleles at the biochemical level of the plasma membrane. In 1985, Albert and Therrien further provided evidence of differences in the plasma membrane composition of induced and uninduced cells by exhibiting a progressive increase in the DNA content of mated induced myxamoeba in comparison to mated uninduced myxamoeba. Therefore, this would suggest that in the induction period indicated by Yemma and Perry (1985), the plasma membrane underwent significant changes which precluded recognition of compatible mating types and subsequent fusion.

This study focused on plasma membrane changes between fusion competent (induced) and pre-fusion (uninduced) myxamoeba of **D. iridis** HON 1-7 A^2 mating strain. Ross et al. (1973) and Yemma et al. (1974) postulated that changes could be affected in the plasma membranes of sexually incompetent myxamoeba by an unknown chemical-inducing substance and in addition, physical contact between cells was also thought to exert some type of control on fusion. In 1991, Yemma and Stroh verified this information by providing evidence concerning the presence of an inducer molecule. It was also suggested that once myxamoeba enter into the induction period, the process of fusion is under genetic control as those genes responsible for affecting changes in the cell surface become expressed (Ross et al., 1973). Furthermore, if fusion does not occur or if contact between cells is not maintained, the myxamoeba are usually subject to lysosomal

destruction (Ross et al., 1973).

Summing up the evidence concerning membrane fusion and applying it to gametic fusion in **D. iridis**, several conclusions can be drawn. Membrane fusion in this myxomycete does require both chemical induction and physical contact and this has been shown extensively in the literature. In mixing together compatible myxamoeba, each at a critical cell density, zygote formation does not immediately occur (Yemma and Perry, 1985). Instead, a chemical inducer molecule released by compatible myxamoeba (Yemma and Stroh, 1991) induces compatible cells whose membranes are less than 10 A^0 apart (Poste and Allison, 1971) to undergo a period of induction (Yemma and Perry, 1985), prerequisite to fusion. During this induction period, the plasma membrane undergoes dynamic changes. Membrane macromolecules become less ordered and conformational changes take place thus permitting interaction with the membrane with which contact has been established. These changes possibly accompany the production of membrane fusion factors. Evidence suggesting this was provided by Albert and Therrien (1985) in their quantification of DNA in both fused induced and fused uninduced myxamoeba in **D. iridis**. Once induction period was concluded and inter-membrane linkages were established, fusion was complete and the newly formed membrane returned to its original stable configuration. If fusion did not occur, though, the unfused cells were destroyed by lysosomal enzymes (Ross et al., 1973). Ross et al. (1973) also suggested that fused cells secrete a

factor that inhibits fusion among haploid cells. This would create a high-efficiency feedback system in which newly formed zygotes feed on unfused haploid myxamoeba, but verification of this has not yet been shown in the literature.

The initial postulation that induction results in the expression of a single membrane fusion receptor seems to be supported by the HPLC analysis of induced and uninduced plasma membrane fractions in this study. HPLC analysis exhibited a common peak at 3.12 +/- .09 minutes in both induced and uninduced, fractions suggesting that the protein represented by this peak is not directly affected during the membrane changes which occur during the period of induction (Yemma and Perry, 1985). The two peaks present in uninduced runs (at 11.20 +/- .09 and 14.83 +/- .09 minutes, respectively) yet absent in induced runs appear to represent those membrane glycoproteins that undergo conformational changes during the restructuring of the plasma membrane surface during this induction period. In addition, the protein peak eluted in the induced runs (at 12.06 +/- .27 minutes) arises in one of two ways. The most likely mechanism is the expression of a membrane surface glycoprotein through conformational changes of proteins already present on the surface. Accompanying the expression of this protein would be the repression of the two proteins which are eluted by uninduced membrane fractions. Results presented in Figures 5 - 6 provide evidence supporting this potential mechanism as the two protein peaks which are eluted during HPLC analysis of uninduced membrane fractions

are lost in the analysis of induced membrane fractions. Only the protein peak at 12.06 minutes is produced by induced membrane fractions. A less likely, although possible, mechanism suggests that the induced membrane glycoprotein fusion factor arises from a hybridization of the two proteins already present on the uninduced membrane surface, represented by the peaks eluted at 11.20 and 14.83 minutes, respectively, as illustrated in Figure 6. Support for the latter mechanism comes from the fact that the protein peak eluted by induced membrane fractions at 12.06 minutes lies between the two peaks produced by uninduced membrane fractions at 11.20 and 14.83 minutes, respectively. Because of the short duration of the induction period during which reorganization of membrane surface molecules occurs, both mechanisms appear possible.

The actual mechanism of membrane fusion in D. iridis continues to be of great interest. Studies are presently being undertaken concerning the identity of the glycoproteins involved in fusion as well as the mechanism by which these mating factors are activated. This could further lead to evidence concerning the interaction of these molecules with one another to allow for formation of a diploid zygote.

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