CHEMISTRY AND PHYSIOLOGY OF AN INDUCED PLASMA MEMBRANE CELL FUSION RECEPTOR IN THE MYXOMYCETES Didymium iridis

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ABSTRACT

Chemistry and Physiology of an
Induced Plasma Membrane Cell Fusion Receptor
in the Myxomycetes, Didymium iridis

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Cellular fusion is a relatively poorly understood phenomenon. Our efforts to help elucidate membrane components involved in cellular fusion have led us to utilize a model system consisting of haploid myxamoebae of the species Didymium iridis. Myxamoebae have shown an ability to undergo fusion with cells of the opposite mating type upon obtaining a critical cell density of 1x10 E+5 cells/ml. At this critical density, myxamoebae produce a substance, similar to trisporic acid, that induces the plasma membranes of the cells in such a way as to allow fusion to occur. The resultant fusion yields diploid zygotes which later give rise to free flowing plasmodia. Our research centers on the differences shown between the plasma membrane proteins of the induced (competent to fuse and mate) and the uninduced (incompetent to fuse and mate) haploid myxamoebae. Our laboratory has made antibodies against the induced myxamoebae. Isolated plasma membranes used in conjunction with the aforementioned antibodies have allowed us to form Ab-cell surface protein complexes. Complex formation has been determined by HPLC, electrophoresis, and fluorescence microscopy.

Examination of these complexes has allowed us to ascertain which cell surface proteins (glycoproteins) are apparently involved in the process of cellular fusion between haploid myxamoebae of D. iridis.

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CHAPTER I

INTRODUCTION

Slime molds or myxomycetes have been used in scientific studies dating back to the 19th century by the German mycologist Anton de Barey (1860). Their short life-cycle exhibiting diverse developmental changes coupled with the relative ease in maintaining myxomycetes in laboratory cultures makes them an excellent eukaryotic model system. Myxomycetes are grouped into two major categories: cellular and acellular. The acellular slime mold Didymium iridis was utilized throughout this study. It demonstrates both a haploid and a diploid phase during its life-cycle (Ross, 1967). The haploid phase consists of the myxamoebae which live on solid media and the flagellate swarm cells, formed by the conversion of myxamoebae, which predominate in an aqueous environment. Plasmodia, multi-nucleated protoplasmic streaming structures, represent the diploid phase of the life-cycle. All forms rely on bacteria as a food source.

Some myxomycetes are self fertile or homothallic. Another condition rarely seen (approximately 1 in 1000 cultures) is apogamy, where the nuclei of myxamoebae divide mitotically, but cytokinesis does not follow, and eventually a plasmodium is formed. Karyogamy and meiosis never occur.

However, D. iridis is heterothallic and must be crossed with cells of a compatible mating type incorporating a single locus

multiple allelic mating system (Yemma and Stroh, 1991). The basis for the mating system arises from the genetic differences in the respective types. Plasma membrane fusion in D. iridis and the eventual production of zygote and plasmodium is controlled as stated by a multiple allelic system which requires the presence of two different alleles at the mating loci. The normal heterothallic mating in D.iridis usually results when two cells of opposite mating types are brought together and undergo syngamy and karyogamy respectively (Collins and Ling, 1972; Collins, 1976). Resulting from the cellular fusion is a zygote possessing the diploid number of chromosomes (Yemma and Perry, 1985). Arising from the zygote, a plasmodium develops which gives rise to fruiting bodies that via meiosis produce spores to complete the life-cycle which normally takes 7-8 days (Figure 1A).

This study focuses on the portion of the life cycle where the haploid myxamoebae and swarm cells of opposite mating types fuse to form zygotes, our particular interest is in the state of the plasma membrane before and after myxamoebae are competent to mate. Cell fusion based on contact between myxamoebae cells of opposite mating type has been shown to occur in D. iridis (Ross et. al, 1973). This fusion mechanism results in the production of stable zygotes (Yemma and Therrien, 1974). Central to fusion taking place is the close apposition and eventual interactions between respective plasma membranes. The plasma membrane has been an interesting area of study since its bi-layer nature was determined in the early 1920's by Gorter and Grendel. The ideas about the structure of the

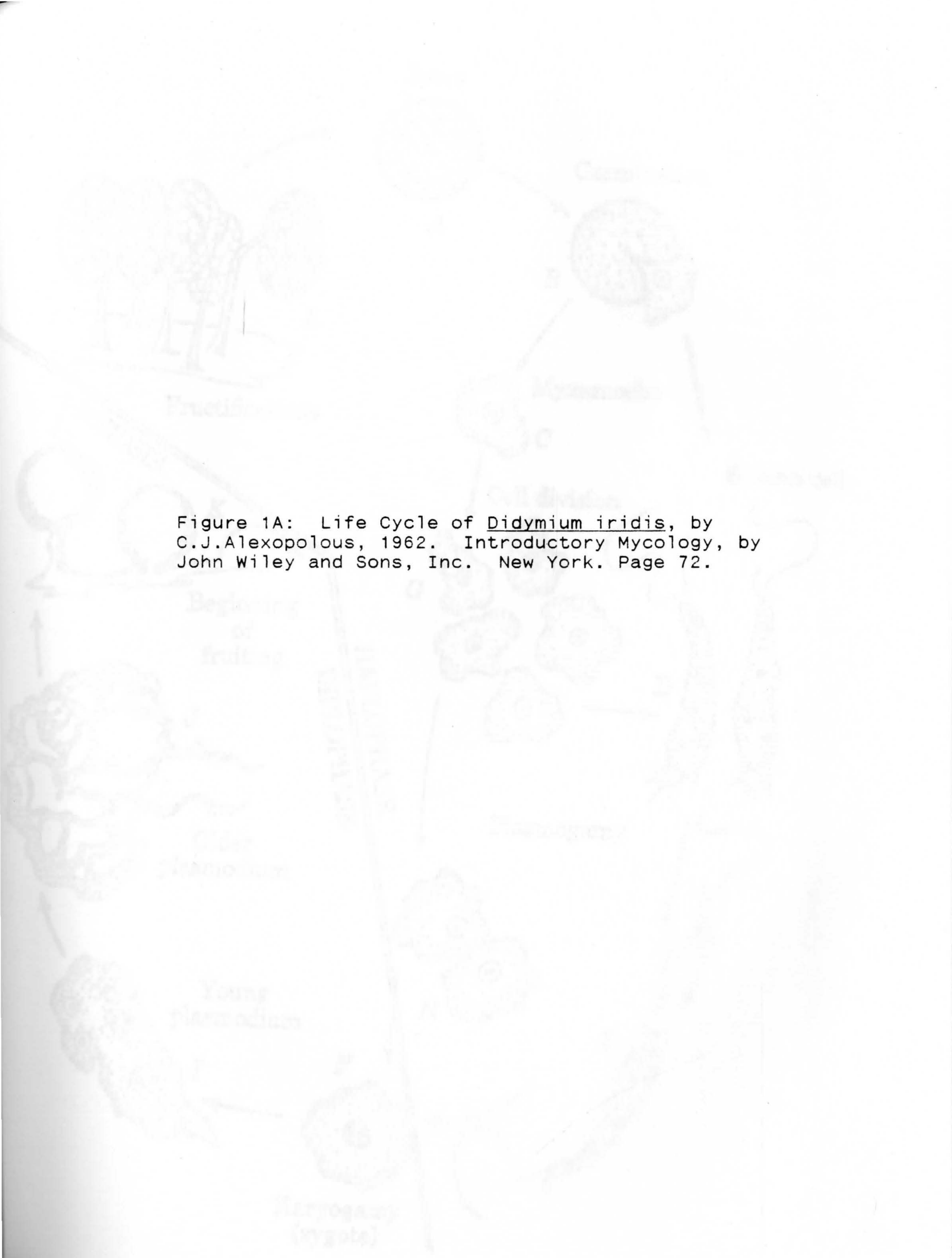
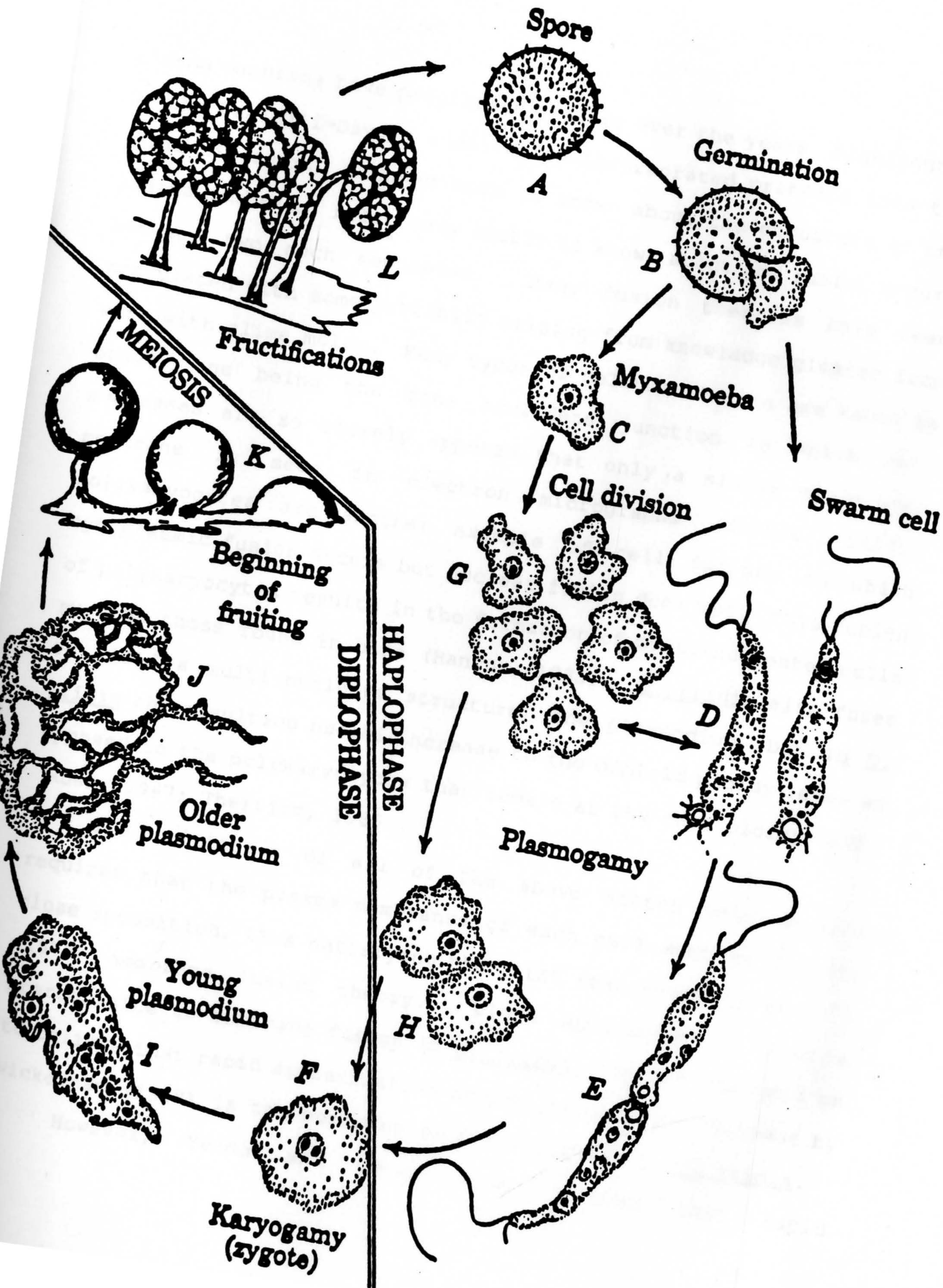


Figure 1A: Life Cycle of <u>Didymium iridis</u>, by C.J.Alexopolous, 1962. Introductory Mycology, by John Wiley and Sons, Inc. New York. Page 72.


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

plasma membrane have progressed greatly over the years, highlighted by the Danielli-Davson model which incorporated proteins into the lipid bi-layer. Although much is known about the structure of the plasma membrane, relatively little is known about how fusion occurs between two such membranes. Many fusion theories have been postulated with some partially arising from knowledge gleaned from work with slime molds. Many types of cellular fusion are known to occur, one being the zona occludens junction in which cell membranes are so closely apposed that only a single bi-layered membrane is seen in electron micrographs (Curtis, 1967). Polykaryocytes are another example of cell fusion, in which cytoplasmic fusion occurs but nuclear fusion does not. This fusion of polykaryocytes results in the formation of multi-nucleated cells such as those found in bone (Hancox, 1965). D. iridis also fuses to yield a multi-nucleate structure, the plasmodium, but in D. iridis the resulting nuclei increase to the diploid ploidy level as opposed to the polykaryocytes that remain at the same ploidy level (Ross, 1967; Therrien, 1966).

The initiation of all of the above stated cell fusions requires that the plasma membranes of each cell must be in very close apposition, thus satisfying the first requirement of Poste's (1970) membrane fusion theory. Close apposition is also a prerequisite to membrane fusion in D. iridis, which could lead us to believe that rapid agglutination as previously shown in yeast by Wickerham (1958) is the membrane fusion mechanism in D. iridis.

However, Yemma and Perry (1985) showed that rapid

agglutination was not taking place in D. iridis by demonstrating that diploid level DNA did not arise immediately after mixing myxamoebae of opposite mating types as would have been expected if agglutination had taken place. In fact, Yemma and Perry (1985) showed that a significant lag time of 20-30 minutes was required to produce diploid DNA. These findings support the idea that membrane-bound mating factors or receptors are involved in the mating cell fusion process of D. iridis (Ross, 1967; Yemma and Perry, 1985).

Of the three major classes of molecules comprising the plasma membrane: lipids,proteins, and carbohydrates, it was demonstrated by Shipley and Ross (1978) that various proteases were able to disrupt membrane proteins and prevent fusion from occurring in D. iridis. The protein involved in fusion was thought to be a glycoprotein because of work done by Molday et al. (1976) and Gillete et al. (1974) which demonstrated that glycoproteins were widely distributed on the plasma membrane of another slime mold, Dictyostelium discoideum. Overwhelming evidence was then presented by Yemma and Soltis (1988) that showed that the lectin Concanavalin A was able to prevent mating fusion from occurring between opposite mating types of myxamoebae. The only exception in this work was that occasionally, less than 2% of the time, plasmodia would develop without the introduction of myxamoebae of an opposite mating type. Upon further analysis these plasmodia were found to have developed by apogamy and are referred to as "selfers". These self forming plasmodia were also found to only contain a haploid

value of DNA (Yemma and Therrien, 1972; Yemma et al., 1972). Selfing myxamoebae are unable to fuse with cells of the opposite compatible mating type and are not thought to possess a mating fusion receptor. From Yemma and Soltis' work it is now believed that a glycoprotein or glycoproteins containing alpha-mannopyranase and alpha-glucopyranase residues are involved in the membrane fusion process since Concanavalin A has been shown to bind preferentially to these sugar moieties. This assessment of the membrane fusion process involving glycoproteins seems reasonable in light of the fact that a glycoprotein referred to as gp80 has been found to play a central role in cell to cell adhesion in Dictyostelium discoideum (Siu, 1990).

Another interesting factor that needs to be taken into consideration is that D.iridis myxamoebae are not always competent to undergo mating fusion. When myxamoebae first arise from spores or emerge from encystment, which is a condition brought about by starvation in which cells form a thick protective covering to prevent desiccation and cellular death, they are unable to fuse with cells of the opposite compatible mating type. Apparently cells must undergo an induction process in which the membrane is altered, as suggested by Poste (1970), to undergo the fusion process. This induction process long hypothesized in D. iridis was thought to occur when cells reached a critical cell density at which time they become competent to mate (Shipley and Holt, 1985; Alberts and Therrien, 1985). The critical cell density at which mating competency is achieved was found to be 1x10 E+5 cells/ml by

Yemma and Perry (1985). Cell density as opposed to nutritional concerns was shown to be the overriding factor in achieving mating competency because Ross et al. (1973) demonstrated that the rate or amount of mating fusion was totally independent of the amount of bacteria available to the myxamoebae as a food source.

When the critical cell density is achieved during the logarithmic growth phase, it was postulated by Youngman et al. (1977) and Pallotta et al. (1979) that an extracellular molecule (referred to as an inducer) was causing the plasma membranes of the myxamoebae to change in such a way as to become competent to fuse with cells of the opposite compatible mating type. The inducer molecule was later isolated by Yemma and Stroh (1991) and was found to be very similar to trisporic acid which was shown by White et al. (1985) to be a maturation hormone in the mucoraceous fungi. The ability of this inducer substance to induce mating competency in D. iridis was shown when this molecule was added to cultures of myxamoebae that were not competent to mate (uninduced) by inducing mating competency in a short period of time (Yemma and Stroh 1991).

Another interesting facet of Yemma and Stroh's work was that myxamoebae cells bring about their own induction and do not need to be in the presence of cells or molecules of the opposite mating type. Yemma and Stroh (1991) also found that the induced cells are only able to form the mating fusion receptors during G1 of the interphase portion of the cell cycle. Thus, the elucidation of the inducer molecule strengthens the hypothesis of Collins and Ling (1968) that states that the mating locus functions as a regulator

gene that is either depressed or corepressed depending on the presence or absence of an inducer substance.

The idea that this inducer works on the genetic level seems reasonable in light of the Central Dogma and the findings of Restivo (Unpublished Thesis) that show different membrane protein profiles exist between induced (competent to mate) and uninduced (incompetent to mate) isolated plasma membranes of D. iridis. It is the hope of this study to further characterize these protein profile differences and to reveal their roles in the plasma membrane mating fusion process.

CHAPTER II

METHODS AND MATERIALS

Culturing of cells

Stock cultures of isolets of D. iridis are maintained in our laboratory on 1/2 strength cornmeal agar slants in wide mouth screw top culture vessels. This study used primarily strain HON1-7A2, but also made use of strain PAN2-21A7B as the compatible mating type. The isolets were originally provided by Dr. O.R. Collins, Department of Botany, University of California, Berkeley.

Preparation of 1/2 strength cornmeal agar:

1. 8.5 grams of Difco cornmeal agar is added to 8.0 grams Difco Bacto-Agar and mixed with 1 liter of distilled water.

2. Solution is then autoclaved at 121 degrees Centigrade at 15 P.S.I. for 15 minutes.

3. 10ml aliquots were then placed into sterile petri dishes (Collins, 1963; Yemma et al., 1974)

After solidifying, plates were inoculated with Escherichia coli bacterium via a flamed alcohol sterilized glass rod. Bacterial lawns were permitted to develop for 24 hours at which time approximately 2cm square agar blocks containing either encysted myxamoebae cells or spores from stock cultures were placed on the inoculated media surface. All culturing was done in a laminar flow hood to minimize risk of contamination. Growth of the myxamoebae was monitored by an Olympus CK2 inverted microscope. Any plates showing signs of contamination were immediately discarded.

Uninduced cells

Myxamoebae arising from either spores or encystment are unable to undergo mating fusion for approximately their first 48 hours, due to the fact that they have not reached the critical cell density of 1.0 x 10 E+5 cells/ml, necessary for the effective production of the inducer substance (Yemma and Perry, 1985; Yemma and Stroh, 1991). Therefore, myxamoebae are removed from the surface of culture plates using a rubber policeman and suctioned off via a sterile disposable pipet before they are 48 hours old.

The resulting cells are placed in 40ml centrifuge tubes along with ultrapure millipore water. The suspension is washed 3x by centrifugation (800rpm for 10 min.) in an IEC HN-SII swinging bucket centrifuge to remove the E. coli that remain in the supernatant. The resulting pellet is resuspended in a sufficient volume of millipore water to insure that the concentration does not approach the critical cell density. Concentration of cells is checked with a hemacytometer (American Optical Brightline #1492). The resulting live uninduced cells, must be used immediately and made ready for membrane isolation. The uninduced nature of the myxamoebae was verified by their ability to fuse (or in this case not to fuse with cells of the opposite mating type).

Induced Cells

Myxamoebae were cultured on half strength cornmeal agar as stated previously. Cultures were permitted to grow for 60-72 hrs. before harvesting. The resulting cells were also removed from the plate and washed as stated previously. The final pellet was suspended in 1ml of millipore water to insure that the cellular concentration was in excess of the critical cell density. Concentrated cells, after being counted on a hemacytometer were allowed to stand for 1.5 hours to insure that induction had taken place. Induction was verified by looking at zygote formation crossing with cells of the opposite compatible mating type (Yemma

and Stroh, 1991). Again, induced cells had to be used immediately or be readied for the membrane isolation.

Membrane Isolation

Isolating the plasma membrane was the primary prerequisite in beginning to examine the proteins present in it. The technique of using a sucrose gradient coupled with ultracentrifugation was chosen for the isolation because it was thought that this would cause the least amount of disruption in the glycoproteins being examined.

The following technique was developed in our laboratory:

1. The induced and uninduced pellet preparations were resuspended in millipore water and loaded into 50ml polyallomer centrifuge tubes and these bacteria-free cells were centrifuged at 2000rpm for 10 minutes in an IEC BA-20 refrigerated centrifuge.

2. Pellets were then twice more washed in .14M NaCl at the above conditions.

3. Clean pellets were then suspended in 15ml of homogenization buffer consisting of 1mM ZnCl2 and 10mM

Tris-HCl (pH = 8.0) in a 1:1 ratio. Cells remained in the homogenization buffer for 15 minutes at room temperature.

4. Centrifuge tubes were then partially immersed in an ice bath to cool the cells to 4 degrees Centigrade. All remaining steps were carried out at 4 degrees Centigrade.

5. Cells were sonicated for 5 to 6 seconds at a constant frequency produced by a Branson Sonifier 250 fitted with an Ultrasonics Converter.

6. Disruption of myxamoebae was confirmed by microscopic inspection of wet mount slides.

7. Fragmented cells were then centrifuged at 14,000rpm for 15 minutes in a Beckman L7-35 refrigerated ultracentrifuge using a fixed angle rotor of type 42.1.

8. The resulting supernatant was discarded, while the remaining pellet was resuspended in 7.5ml 10% (w/v) sucrose solution.

9. The sucrose suspension was sonicated at a constant frequency for one second.

10. 5ml of the sucrose suspension was then loaded onto a 20% to 50% (w/v) linear sucrose gradient composed of 6ml aliquots per gradient. The gradients were housed in 30ml Oak Ridge screw-on cap style ultra centrifuge tubes.

11. Gradients were centrifuged at 26,000rpm for 2 hours in the ultracentrifuge.

12. Fractions were carefully removed in approximately 2ml aliquots by inserting tygon tubing to the bottom of the 50% sucrose layer. The tubing was connected to a New Brunswick Scientific peristaltic pump (model M1062) which was variably controlled by a Powerstat 10amp autotransformer. The peristaltic pump was used to suck out the fractions and place them in test tubes contained in a BIO-RAD model 2110 fraction collector.

13. The resulting aliquots were then tested to see which fractions contained the highest amount of the plasma membrane marker enzyme 5' nucleotidase. The assay performed was taken from Evans (1978) and the inorganic phosphate was quantified by the method of Chen et al. (1956).

Note: After many trials our laboratory determined that the highest concentration of 5' nucleotidase always

occurred in the 40% sucrose gradient (between 6 and 12 mls from bottom of centrifuge tube). Therefore, this 6ml fraction is used as our isolated membrane fraction in all subsequent experiments (Restivo, thesis 1992).

Concentration of Membrane Proteins

The 6ml isolated membrane fractions were poured onto sterile watch glasses which were then covered with parafilm. After small holes were poked into the parafilm, the watch glasses were placed into the drying chamber of a Labconco Freeze Drying System. The samples were frozen at -60 degrees Centigrade. The system was then vacuum sealed to a pressure of less than 10 microns Hg. The set point of the system was raised to 2-4 degrees Centigrade. Lyophilization was allowed to occur until sample appeared dry (approximately 24-36 hrs.). The resulting membrane and sucrose crystals were removed from the watch glass by adding 300ul of millipore water. The resulting concentrated suspension was placed into cryotubes and immersed in liquid nitrogen for long term storage.

Preparation of Antibody Against Induced Cells of D. iridis

The following technique for antibody production was developed in our laboratory for D.iridis:

Cells of D. iridis strains CR5-5A2 and CR5-11A11 were raised in a liquid culture containing PBS buffer media along with formalin killed E. coli. The bacteria were killed in the hope of limiting the possibility of septicemia occurring in the test animals. Cells were grown to a density of 1.0 x 10 E+6 cells/ml and then harvested. Next, cells were washed three times in PBS by centrifugation at 150xG to remove any E. coli present. The resulting 40ml suspension was then divided into 4 equal volumes, with each aliquot being placed in a glass parabiotic chamber and oxygenated with an aquarium pump for 4hrs. to insure induction. After incubation, cells were twice more washed in PBS leaving four 1.5ml suspensions of each strain. One of the four aliquots from each strain was mixed 1:1 with Freunds Complete Adjuvant, to later be used for the first injection. The remaining three aliquots of each strain were mixed 1:1 with Freunds Incomplete Adjuvant which was used for all later injections.

Injection Procedure

Two test rabbits were tranquilized with Acepromazene

(.25ml/lb.) and injected intradermally along the back in .25ml aliquots of the cell preparations. Each injection contained approximately 8.0 x 10 E+7 cells of a single mating type, either A2or A11. Three separate injections were given with a three week period between injections.

Conformation of Antibody Production

Two weeks after the second injection, a 10ml blood sample was taken from each rabbit. Blood was allowed to clot at room temperature for 4 hours, and then centrifuged 3x at 150xG. Supernatant containing serum was collected, while pellet was discarded. Two drops of serum were then mixed with two drops of live myxamoebae on a slide and observed with a phase contrast microscope. Cells were seen to clump and lyse within 20 minutes when exposed to the serum. The resulting clumping and lysing observed was in stark contrast to the lack of any action occurring when cells were mixed with control serum (collected from rabbits prior to any injections). The clumping and lysing indicated that a positive Antibody titer was present. Three weeks after the last injection, the rabbits were sacrificed via cardiac puncture in order to obtain more antibody. The A2 antibody described in this section was used extensively in subsequent experiments and will be referred to as Anti-A2.

Visualization of Antibody Binding

1. 200ul of bacteria-free uninduced and induced cells were placed on different halves of a glass slide.

2. Anti-A2 was heated to 56 degrees Centigrade for 20 minutes to remove complement.

3. Anti-A2 was then diluted 1:128 with millipore water.

4. 2ul of anti-A2 was added to each set of cells on the slide and mixed with a pipet tip.

5. 2ul of Goat anti-Rabbit IgG heavy and light chain labeled was added to Fluorescein isothyocyanate (FITC) purchased from Cappel Laboratories; Cochranville, PA.

6. Slides were then incubated at 37 degrees Centigrade for 1/2 hour.

7. Slides were viewed with an Olympus BH-2 microscope fitted with a fluorescent power pack.

8. Photographs were taken with an Olympus C-35AD-4 camera using Kodak 160 Tungsten Ektachrome film (See Plates 1-6).

Anti-A2 fusion blocking experiment

It was previously noted by our laboratory that heating Anti-A2 serum to 56 degrees Centigrade for 20 minutes destroys complement, which allows the Anti-A2 to bind to the cell membrane without causing lysing.

1. Plates of PAN2-21A7B were grown for 72 hours as described previously.

2. Complement was removed from Anti-A2. Control serum was treated in a similar fashion.

3. 150ul of HON1-7A2 induced myxamoebae were then mixed in a test tube along with 6ul of Anti-A2. Another tube utilizing the Control-A2 serum and induced cells was also prepared.

4. Both tubes were incubated at 37 degrees Centigrade for 30 minutes.

5. Two sets of plates were created by applying 15ml aliquots of the solutions to the PAN2-21A7B lawns of the opposite mating type.

6. Plates were examined daily for 8 days with an inverted microscope to ascertain if any plasmodia had developed.

Electrophoresis

After previously failing to migrate proteins, it was decided to utilize the charge bearing properties of Sodium Dodecyl Sulfate (SDS) to assist in the migratory process.

Sample Preparation

The lysing solution consists of 7.5ml of .05M Tris-HCl buffer adjusted to pH = 7.0 by addition of 1N HCl mixed with 0.2g SDS (Sigma), 2.0mg bromophenol blue (tracking dye), 2.0g sucrose and 0.5ml Beta-mercaptoethanol. The resulting lysing solution was mixed 1:1 with induced and uninduced membrane fractions. Anti-A2 and Control-A2 were first rid of complement and then diluted 1:128 with millipore water, before being added in equal volumes with SDS. Six different samples were run:

1. UN(10ul)+ SDS(10ul)

2. IN(10ul)+ SDS(10ul)

3. UN(10ul)+ Anti-A2@ 1:250(10ul) + SDS(20ul)

4. IN(10ul)+ Anti-A2@ 1:250(10ul) + SDS(20ul)

5. Anti-A2@ 1:128(10ul) + SDS(10ul)

6. Control-A2@ 1:128(10ul) + SDS(10ul)

After preparation, the above samples were heated to 65 degrees Centigrade to denature proteins and allow SDS to bind and impart its negative charge.

Electrophoretic Parameters

Two electrophoresis kits were utilized in this study:

1. Titan Gel High Resolution Protein Kit Cat.# 3040 by Helena Laboratories Beaumont, Texas

2. Titan Gel Silver Stain Kit Cat.# 3035 by Helena Laboratories

Both kits contained similar sodium barbital buffers (pH = 8.4-8.8) which were used with their respective gels. Both kits also have the same running parameters and sample application procedures. The

only deviation taken in the included protocol was to increase the sample size from 4ul to 8ul.

The kits were run in the following manner:

1. Gels were removed from packages and lightly blotted in the area where the samples are to be applied.

2. Template is aligned between the (-) signs.

3. Six samples of 8ul were added and allowed to absorb for 5 minutes.

4. Template is blotted with blotter from step 2 then removed.

5. Gels were positioned on top of cooled glass plates resting on crushed ice in an Helena electrophoretic chamber connected to a Titan power pack (Helena).

6. Wicks were placed between the gel and buffer to provide contact.

7. Gels were run at 250V for 25 minutes.

At this point each kit follows its own staining protocol.

Coomassie Blue Stain

1. Gel is immediately placed in Coomassie Blue (Coomassie Blue with 500ml Methanol, 100ml glacial Acetic acid and 500ml millipore H2O) after running.

2. Wash gel in 500ml Methanol with 100ml glacial Acetic acid and 500ml millipore H2O.

3. Dry gel in a convection oven till dry at 60-70 degrees Centigrade.

4. When dry swirl gel in wash solution from step number 2 to remove excess stain.

5. Dry gel as in step number 3.

Silver Staining

1. Wash gel for 5 minutes after electrophoresis.

2. Dry gel same a step number 3 above.

3. Fix for 10 minute

4. Wash twice for 5 minutes in accelerator reagent.

5. Wipe back of gel with lint free tissue.

6. Place gel in 100ml of reducing agent for at least ten but no more than 30 minutes.

7. Silver stain for 3-5 minutes.

8. Stop bath for 10 minutes, then dry in oven.

9. Store gel in dark.

<u>Scanning of Gels</u>

Gels were read on an LKB Ultroscan XL Enhanced Laser Densitometer. Data was then integrated on an Everex 386/25 computer utilizing an LKB Gel Scan program.

High Performance Liquid Chromatography

Preparation of samples

Six samples were normally run as a group. Before using the Anti-A2 serum or the Control-A2 serum complement was removed. IN and UN refer to isolated membrane fractions. Samples were placed in a labeled 300ul sample vial with septum and screw-down cap. All samples were filtered through a .45um Arco syringe filter prior to running. Also, all samples were incubated at 37 degrees Centigrade. Samples also contained Acetonitrile (ACN) by (Fisher Chemical) and 0.02M Phosphate Buffer (Phos) made by mixing 438.5ml of 0.02M monobasic sodium phosphate with 61.5ml of 0.02M dibasic sodium phosphate plus 500ml of millipore water to achieve a pH = 8.0. The samples were as follows:

1. UN(10ul) + ACN(20ul) + Phos(20ul)

2. IN(10ul) + ACN(20ul) + Phos(20ul)

3. IN(10ul) + Anti-A2@ 1:500(10ul) + ACN(40ul) + Phos(40ul)

4. UN(10ul) + Anti-A2@ 1:500(10ul) + ACN(40ul) + Phos(40ul)

5. Anti-A2@ 1:250(10ul) + ACN(20ul) + Phos(20ul)

6. Control-A2@ 1:250(10ul) + ACN(20ul) + Phos(20ul)

Running Parameters

15ul samples were injected via a Perkin-Elmer (ISS-100) Auto sampler onto a C-18 Aquapore (220 x 4.6 mm x 7um) RP-300 column from Pierce Chemical Co. Column was contained in a Perkin-Elmer LC-60 Column oven set at 37 degrees Centigrade. Absorbance was detected by a Perkin-Elmer 7500 professional computer. Mobile Phases were filtered daily through a .45um Magna nylon filter manufactured by Micron Separations Inc. attached to a Millipore filtering apparatus. Mobile Phase A: 100% ACN Mobile Phase C: 0.02M Phos Mobile Phase D: ultra pure millipore water

Step 0: 10min equilibration 100% A at 2.0ml/min

Step 1: 25 min run 100% A to 50% A and 0% C to 50% C at 1.0ml/min

Step 2: 10 min wash 100% D

Step 3: END

CHAPTER III

Results

Experiments were designed in order to investigate the differences in protein composition of induced plasma membranes (competent to fuse and mate) and uninduced plasma membranes (incompetent to fuse and mate). Anti-A2 antibody, which was produced against induced cells only, functioned as a differentiator in allowing us to form Ab-protein complexes between Anti-A2 and induced cell surface proteins. Since Anti-A2 was raised only against induced cells, the addition of Anti-A2 to uninduced cells would only form Ab-protein complexes between the proteins common to both the induced and uninduced plasma membranes. It must be emphasized that the Anti-A2 was raised against the entire protein constituency of induced cell membranes, not against an individual protein. Detection of Anti-A2/protein complexes was vital in order to determine whether specific cell surface proteins are involved in the cellular fusion process between the haploid myxamoebae of _D. iridis_.

Before detection of the Ab-protein complexes could be confirmed, experiments had to be designed not only to insure that Anti-A2 was able to bind to induced cells but that it was able to bind to the specific cell surface proteins involved in cellular fusion. The ability of Anti-A2 to preferentially bind induced and not uninduced cells was demonstrated by fluorescence microscopy. By using goat anti-rabbit IgG antibody labeled with a FITC tag it

was possible to visually ascertain by this indirect method of immunolabeling; where and to what degree Anti-A2 binds to cells. As expected, Anti-A2 was found to significantly bind to induced cells (Plates 3-6). Notable binding of Anti-A2 to uninduced cells occurred to a much less appreciable degree (Plates 1 and 2). It should be noted that although immunolabeling can be used to determine induced and uninduced populations from one another; our laboratory still relies, as previously discussed, on the dependable method of cellular concentration counts (Yemma and Perry, 1985). To determine if Anti-A2 forms a complex with the proteins involved in cellular fusion, a fusion blocking experiment was developed. The theory behind the blocking experiment was that if Anti-A2 did indeed bind the relevant cellular fusion proteins then it should be able to prevent cellular fusion and the subsequent formation of plasmodia from developing when cells of the compatible mating type are crossed. Culture plates containing cells of compatible mating types were crossed and marked for plasmodium formation which normally only results after cellular fusion (Yemma and Stroh, 1991).

<u>Anti-A2 Blockage of Cellular Fusion</u>

Only 4 out of 44 (9.1%) plates containing induced cells treated with Anti-A2 antibody underwent cellular fusion between cells of opposite mating types. Cellular fusion was assayed, by the detection of plasmodia that normally only develop after cellular fusion has occurred. In the control plates lacking Anti-

A2 the results were strikingly different; 33 out of 40 (82.5%) plates showed positive results for cellular fusion.

The blocking experiment showed that Anti-A2 was definitely binding to the proteins involved in cellular fusion. When Anti-A2 was added to induced cells only 9.1% of these plates gave a positive result that fusion had occurred. Contrastingly, 82.5% of the plates treated with the control serum lacking Anti-A2 tested positively for the occurrence of cellular fusion. Therefore it can be concluded that Anti-A2 has bound to the fusion receptors and inhibited cellular fusion. The concept of the Anti-A2 being able to block fusion from occurring by binding to the fusion receptor is similar to the experiments performed by Yemma and Soltis 1988 which initially showed that the fusion receptor was a glycoprotein. The results from these initial experiments provided for the development of a method for the actual detection of the Anti-A2/fusion receptor complexes.

To detect these complexes, an instrument displaying the ability to detect molecular level interactions between proteins was needed. The HPLC met this criteria and was selected as the primary analytical tool of this study. Chromatographs were analyzed paying particular attention for any changes in peak retention time upon addition of Anti-A2 to isolated cell membrane fractions. Formation of an Ab-protein complex would lead to an increased retention time of the protein when bound by antibody as opposed to a decreased retention time as shown by the protein alone.

HPLC

In HPLC experiments, precautions had to be taken to prevent any contamination of samples due the extreme sensitivity of the instrument. Glassware was cleaned with 1% nitric acid or the stronger mixture of 20 grams potassium dichromate/250ml H_2SO_4 to prevent the introduction of any extraneous proteins or deleterious proteolytic agents. Acid washed glassware was given a final rinse with ultrapure millipore water. Mobile Phases were filtered on a daily basis to eliminate bacterial and fungal contaminants. All chromatographs in this section were read at an absorbance of 280mm, standard for protein detection.

The chromatograph representing uninduced membrane fractions (Figure 1) displays 3 distinct peaks. Retention times were obtained from averages determined over a series of many trials. Retention time and voltage data for uninduced membrane are listed in table 1.

Table 1

HPLC Data: Uninduced Membrane

Retention Time	Voltage
3.10	1.488
11.25	1.490
14.41	1.488

Time in Minutes
Voltage in Volts

Induced membrane protein profiles are displayed in Figure 2, corresponding data is shown in table 2.

Table 2

HPLC Data: Induced Membrane

Retention Time	Voltage
3.08	1.6900
11.98	1.6930

Time in Minutes
Voltage in Volts

The induced membrane has 2 characteristic protein peaks. The first of which also appears in the uninduced chromatograph (Figure 1). In all HPLC runs undertaken involving only membrane fractions, this "co-eluting" peak is always found.

Upon addition of Anti-A2 to the uninduced membrane, little change emerges in the chromatograph (Figure 3) and resulting data is presented in table 3.

Table 3

HPLC Data: Uninduced Membrane + Anti-A2

Retention Time	Voltage
2.96	1.4883
10.98	1.4904
14.04	1.4875

Time in Minutes
Voltage in Volts

Striking changes occurred upon the addition of Anti-A2 to the induced membrane fraction. The 2 peaks characteristic of the induced membrane undergo a rightward shift showing an increased

retention time. These results are shown in Figure 4 and in table 4. The HPLC data also indicates that a possible conformational change has occurred regarding the membrane proteins allowing it to proceed from an uninduced state into an active induced one. In figure 1 representing the uninduced membrane; the rightward 2 protein peaks apparently hybridize to form the rightward peak observed in figure 2 of the induced membrane. The justification of hybridization occurring is based upon analysis of the retention times of the aforementioned peaks. The two rightward most uninduced peaks have retention times of 11.25 min. and 14.41 min., while the induced peak believed to be the hybridized product of the two uninduced peaks has a retention time of 11.98 min. Thus the induced peak has a retention time which is between both of the retention times for the uninduced peaks. It must also be noted that whereas 3 consistent peaks were recorded for the uninduced cells, only 2 peaks appear in the induced cells. This therefore satisfies the condition that a peak formed via a hybridization would have a retention time between both of the peaks from which it was formed. The induced peak also lies closer in terms of retention time to the larger uninduced peak (11.25 min.) which would have contributed to a greater extent to the formation to the hybridized peak. Because this first uninduced peak contributes more to the hybridized peak than does the second smaller uninduced peak the retention time for the hybridized peak should lie closer to the larger peak. Based on the numerical data in figures 1 and 2 this is found to be the case since the retention time of 11.98

min. lies closer to the larger uninduced peak at 11.25 min. than to the smaller uninduced peak at 14.41 min.

Table 4

HPLC Data: Induced Membrane + Anti-A2

Retention Time	Voltage
5.17	1.4953
15.98	1.4960

Time in Minutes
Voltage in Volts

Figure 1 and Figure 2 demonstrate a curve representing a standard protein peak profile for uninduced and induced isolated plasma membranes. The first peak in Figures 1 and 2 is characterized by retention times of 3.10 min. and 3.08 min. respectively; which are co-eluting peaks found in all profiles of isolated membranes of <u>D. iridis</u>. The remaining two peaks in Figure 1 were present only in uninduced membranes, while the remaining peak in figure 2 was present only in induced membrane preparations. In Figure 3 it should be noted that upon addition of Anti-A2 to the uninduced membranes no significant changes in retention time occurred when compared with the uninduced membrane alone. This finding suggests that strong Ab-protein complexes do not form with the uninduced membrane proteins. Figure 4 shows a definite increase in retention time among peaks upon addition of Anti-A2 to induced membranes, when compared with the peaks present in the induced membranes from figure 2. These changes in retention times

are believed to have resulted from the formation of Anti-A2/fusion receptor complexes. It was observed that retention time increased significantly in the represented two primary peaks when comparing induced (Fig.2) and induced + Anti-A2 (Fig.4) protein peak files. The large shifts in retention time observed are to be expected for it is reasonable to assume that a great number of fusion receptor sites are present in the membrane. This contention is supported by the degree of fluorescence observed in these cells and due to the fact that in order to be involved in a process as vital to the cell as reproduction they would have to be quite numerous. Additional justification for this hypothesis can be supported by the fact that a great deal of adhesion molecules must be present to overcome the hydrophobic barriers produced when two membranes are brought in close proximity and subsequently fuse.

Electrophoretic results of this study were intended to reinforce the results of our findings previously demonstrated in the HPLC data. The use of electrophoresis was limited to this application due to its lower resolving power when contrasted with the HPLC in this case. However, electrophoresis provided valuable insight into the actual elucidation of a major component of Anti-A2.

Electrophoretic Results

During electrophoretic experiments, great care was taken to ensure that gels were not contaminated by any extraneous proteins; therefore gloves and tongs were used during handling of gels.

In all following electrophoretic diagrams, the gel is shown in its entirety, but the scanning densitometer representation displays only the area of interest on the gel. By doing this, an enlargement of the area of interest is represented by the scan. Failing to enlarge this area results in a scan where the peaks of interest are barely discernable.

Distances from the point of application were obtained from data generated by the scanning densitometer and are listed in the following electrophoretic tables. However, these distance from origin calculations rely on the assigning of peak widths and peak locations by the operator; therefore some variability is to be expected in the values listed. Since the peak widths are used in calculating relative area under the curve percentages, some variability is also present in these calculations.

Figure 5 displays the gel and scan of uninduced membrane fractions. Corresponding numerical data is given in table 5.

Table 5

Electrophoresis: Uninduced Membrane

Distance Migrated (mm)	Relative Area %
21.45	16.4
32.85	83.6

Induced membrane gel and scan patterns are shown in Figure 6. Induced membrane data is shown below in table 6.

Table 6

Electrophoresis: Induced Membrane

Distance Migrated (mm)	Relative Area %
22.31	10.2
32.63	89.8

Figure 7 displays that which occurred when Anti-A2 was added to the uninduced membrane. Corresponding numerical data is presented in table 7.

Table 7

Electrophoresis: Anti-A2 + Uninduced Membrane

Distance Migrated (mm)	Relative Area %
24.89	14.8
33.27	85.2

Figure 8 shows the scan and gel when Anti-A2 is added to the induced membrane fraction. Numerical data is presented in table 8.

Table 8

Electrophoresis: Anti-A2 + Induced Membrane

Distance Migrated (mm)	Relative Area %
24.03	39.7
29.62	57.9
32.20	2.4

In comparing control serum with Anti-A2 serum; differences were seen that served to verify the actual presence of Anti-A2, namely an additional band at 17.15mm. Anti-A2 data is shown in Figure and Table 9, while control serum data appears in Figure and Table 10.

Table 9

Electrophoresis: Anti-A2 Serum

Distance Migrated (mm)	Relative Area %
17.15	5.2
27.26	39.6
30.05	12.1
32.63	43.1

Table 10

Electrophoresis: Control Serum

Distance Migrated (mm)	Relative Area %
27.68	45.1
29.19	13.9
32.42	4

The additional high MW band (slow migrating) present in Figure 9 at a migratory distance of 17.15mm not present in the control serum (Fig. 10), is believed to represent a majority of the Anti-A2. As shown previously by the HPLC data, Anti-A2/protein complexes appear to form in an appreciable concentration upon comparing the induced membrane (Fig. 6) to the induced membrane

treated with Anti-A2 (Fig. 8). The addition of Anti-A2, it is reasonable to assume, has effectively prevented the majority of the membrane proteins from migrating as well as those left untreated. The shift in distance migrated from 32.63mm to only 29.62mm for the major peak, lends credence to the assumption of complex formation. The appearance of a rather large peak at a distance of 24.03mm in figure 8 also shows that interactions have occurred between Anti-A2 and the induced membrane. When comparing the uninduced membrane (Fig. 5) with the uninduced membrane + Anti-A2 (Fig. 7), relatively little change is seen as would be expected, when one considers the lower affinity Anti-A2 has for uninduced membranes. Due to the lower resolving power of electrophoresis versus HPLC the additional peak found in uninduced chromatographs is not present when comparing the gels of uninduced (Fig.5) with induced membrane fractions (Fig.6). In actuality little change is seen in the uninduced and induced electrophoretic patterns.

Preservation of Samples

All isolated membrane fractions and antibody samples were stored frozen in liquid nitrogen to prevent adverse changes in the membrane proteins which were the focal points of this study. All isolated fractions were obtained via the isolation procedure of Restivo (Thesis, 1992). This procedure was very useful in obtaining high yields of well purified plasma membrane.

Test for Sexual Competency

The use of cell counts in this study was vital to determine induced and uninduced populations of cells. Cell counts are the easiest and most reliable method used to obtain the state of sexual competency of a population. Although Anti-A2 was originally produced against D. iridis heterothallic isolate CR5-A2, enough homology exist between that isolate and HON-7A2 for the Anti-A2 to have been of great value in the various experiments of this study.

Photographic Results of Anti-A2 Binding

Photographs were taken immediately following the incubation period to avoid deleterious quenching of the FITC tag from occurring. Many repetitions of this experiment were undertaken to provide photographs that were representative of both the induced and uninduced population of cells.

Plates 1 and 2 show real images of uninduced cells. The striking feature here, is how the tagged Anti-A2 complex appears not to be bound to the cells in any appreciable amount.

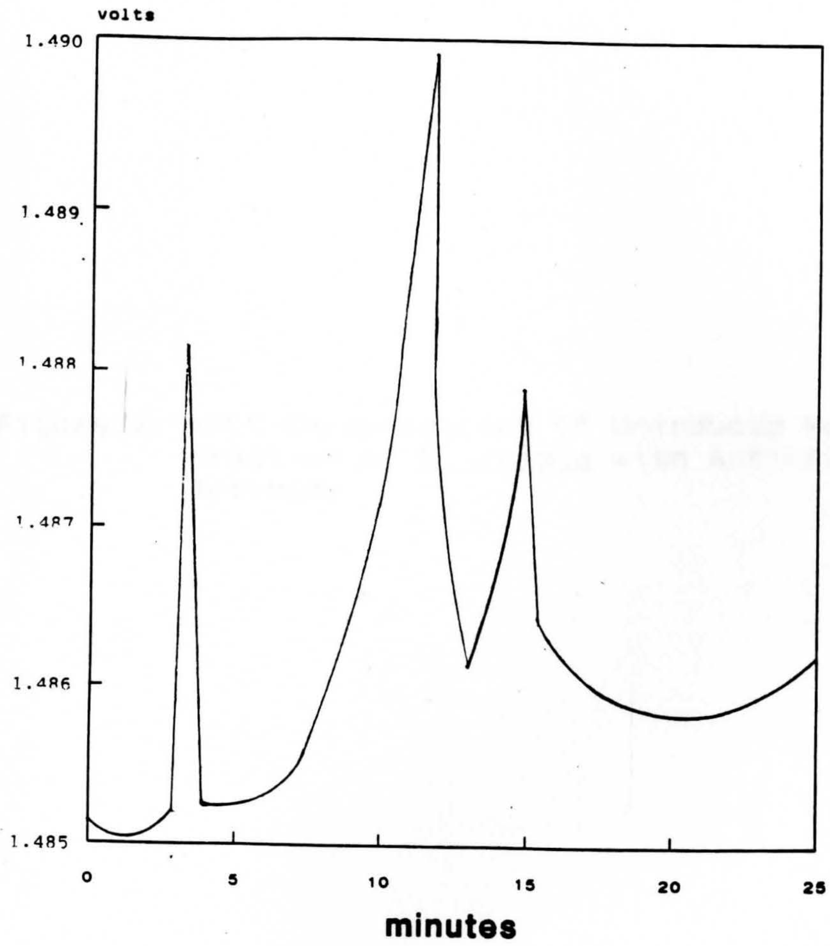
Plates 3 and 4 are negative images of induced cells (i.e. the FITC tag is shown dark, while the normally dark background is shown light). This negative technique was used to bring about the detail of the capping process occurring on the membranes of these cells.

Plate 5 is the real image of the induced cell in Plate 4; note that the capping is not as distinctly seen here.

Plate 6 is a real image of capping occurring equatorially on an induced cell.

Figure 1: HPLC Chromatograph of Uninduced Membrane Fraction of D. iridis

Figure 2: HPLC Chromatograph of Induced Membrane Fraction of D. iridis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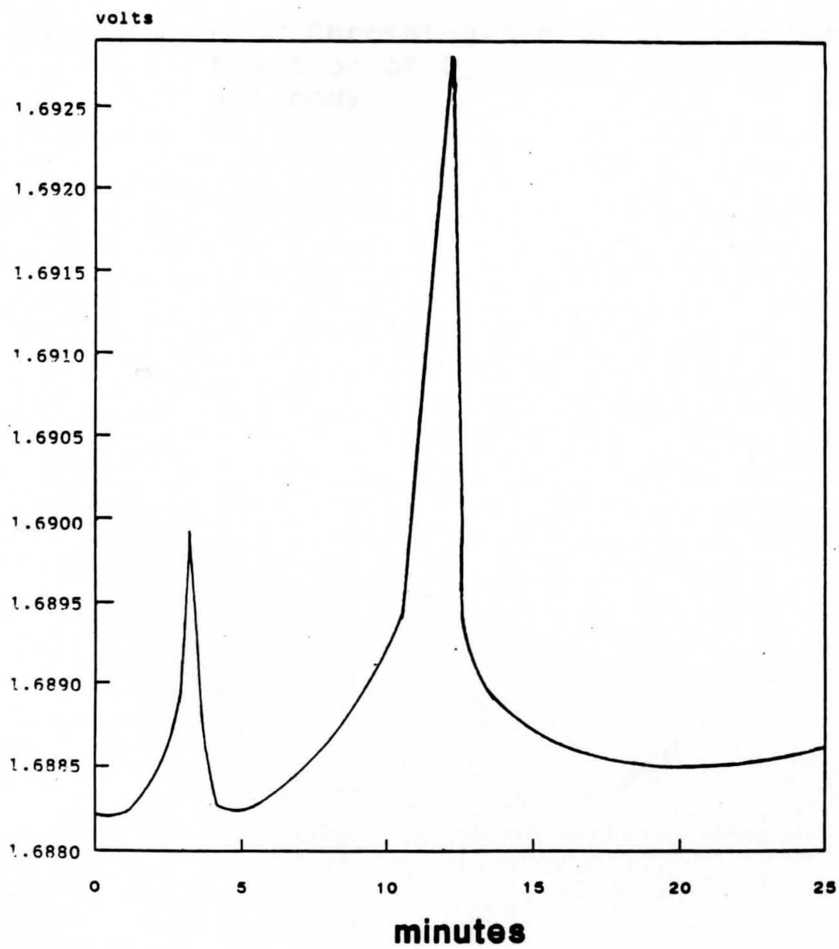
1.489
1.488
1.487
1.486
1.485
0
5
10
15
20
25
minutes


volts
1.6925
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1.6915
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1.6900
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0
5
10
15
20
25
minutes

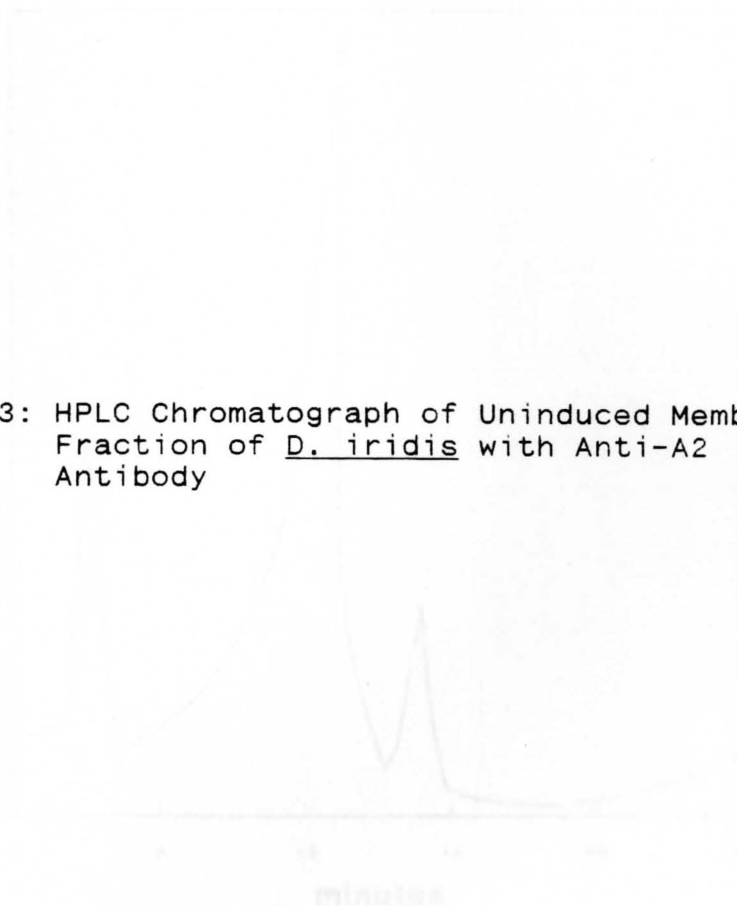
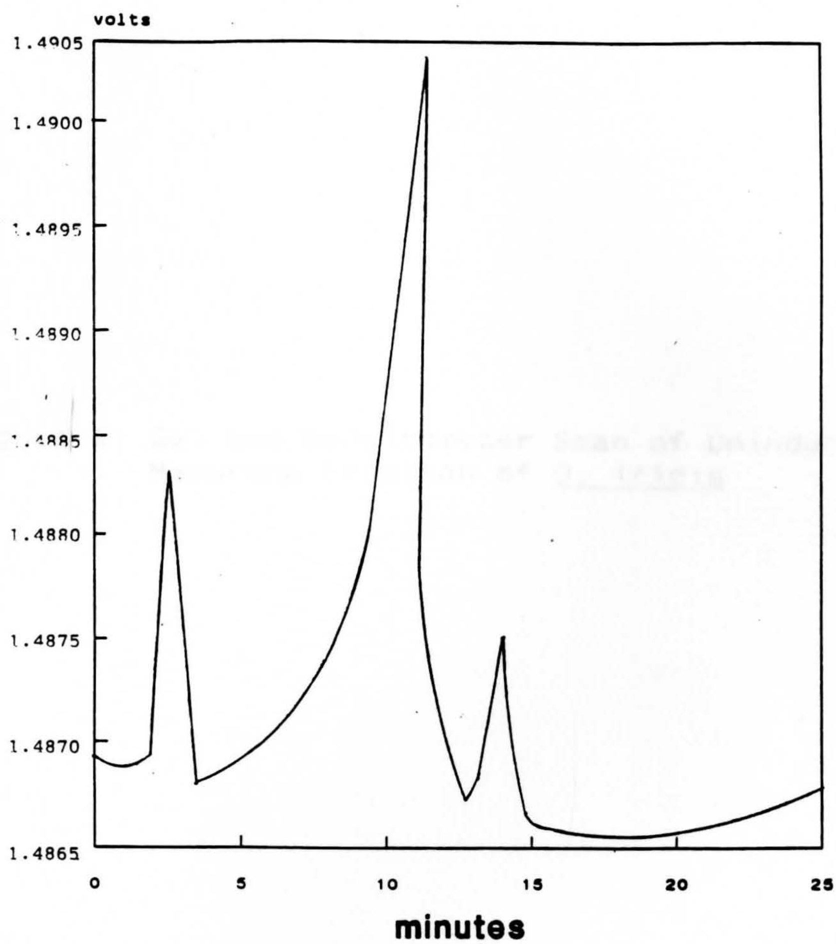


Figure 3: HPLC Chromatograph of Uninduced Membrane Fraction of D. iridis with Anti-A2 Antibody

Figure 4: HPLC Chromatograph of Induced Membrane Fraction of D. iridis with Anti-A2 Antibody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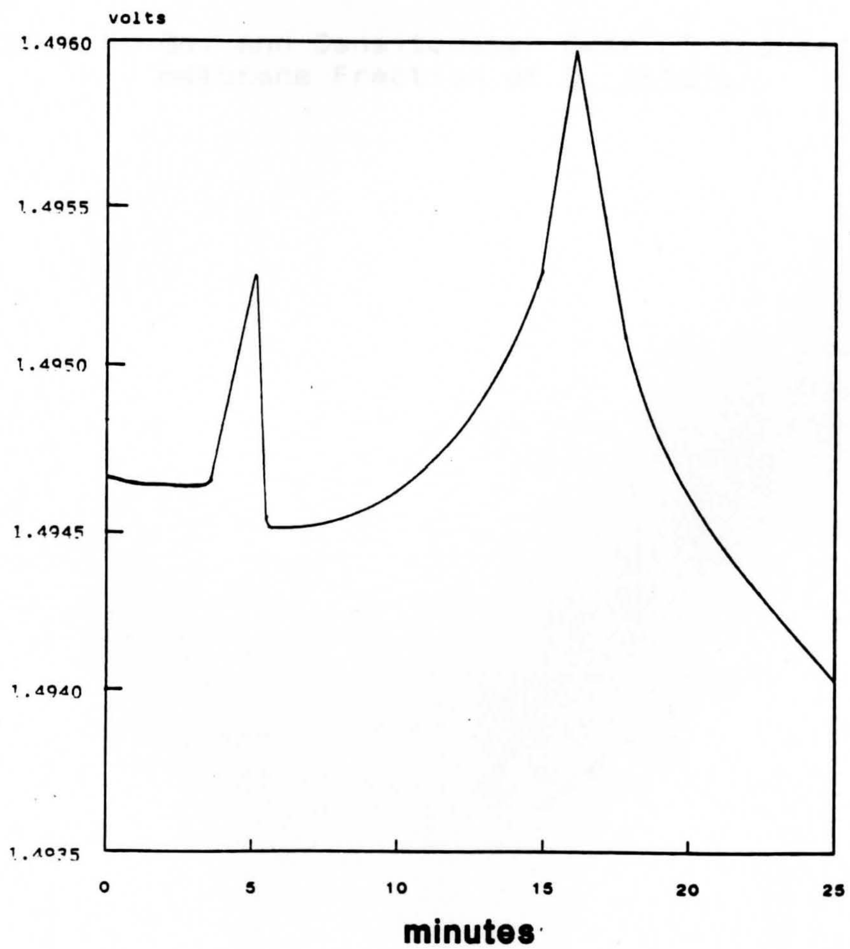
1.4900
1.4895
1.4890
1.4885
1.4880
1.4875
1.4870
1.4865
0
5
10
15
20
25
minutes


volts
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1.4955
1.4950
1.4945
1.4940
1.4935
0
5
10
15
20
25
minutes

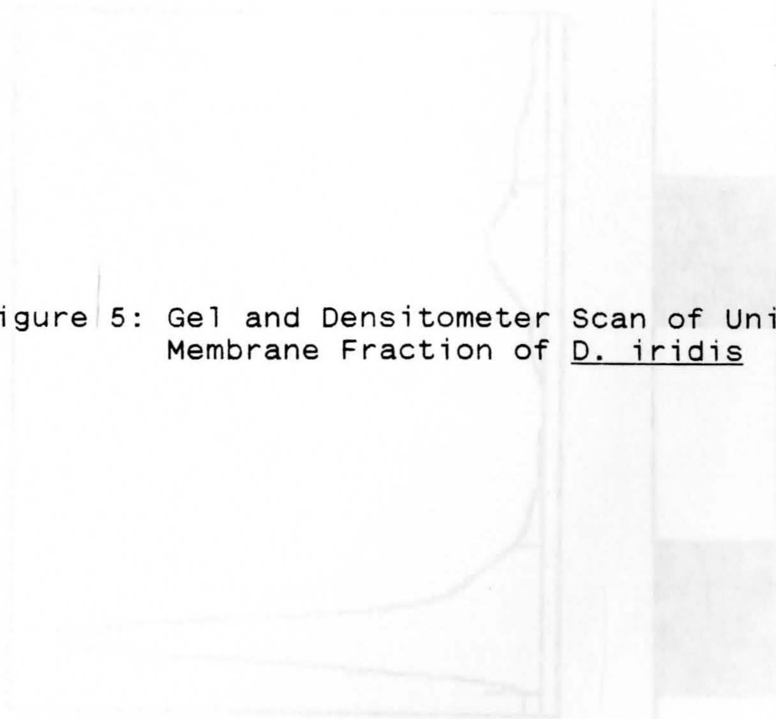
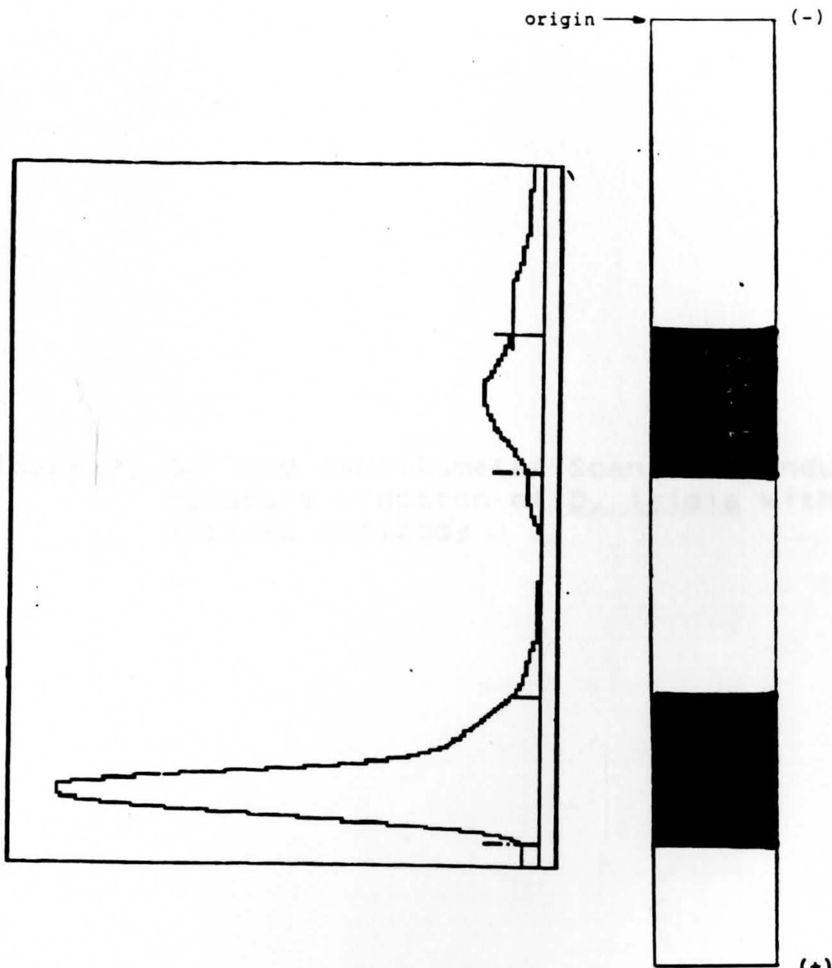


Figure 5: Gel and Densitometer Scan of Uninduced Membrane Fraction of <u>D. iridis</u>

Figure 6: Gel and Densitometer Scan of Induced Membrane Fraction of <u>D. iridis</u>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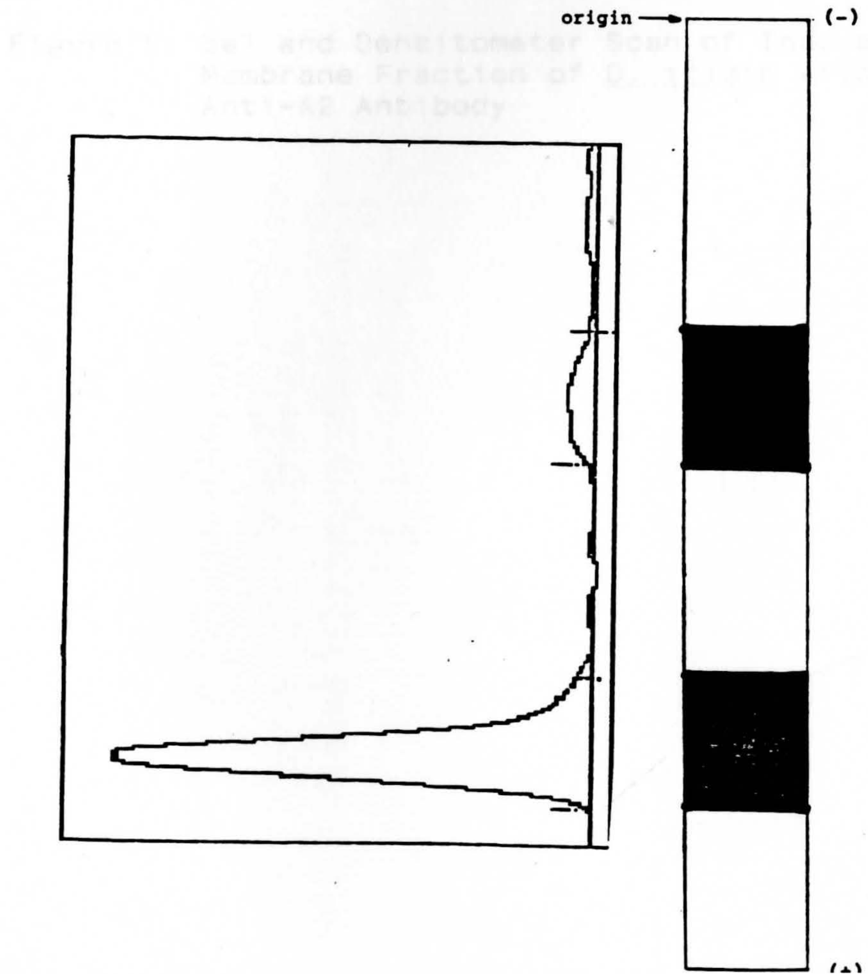
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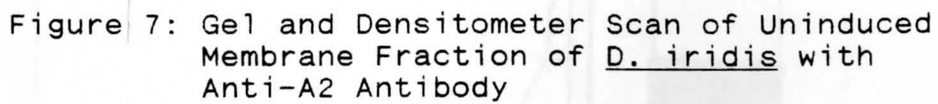


Figure 7: Gel and Densitometer Scan of Uninduced Membrane Fraction of <u>D. iridis</u> with Anti-A2 Antibody

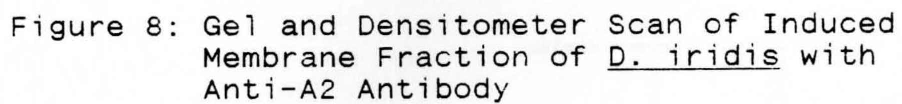
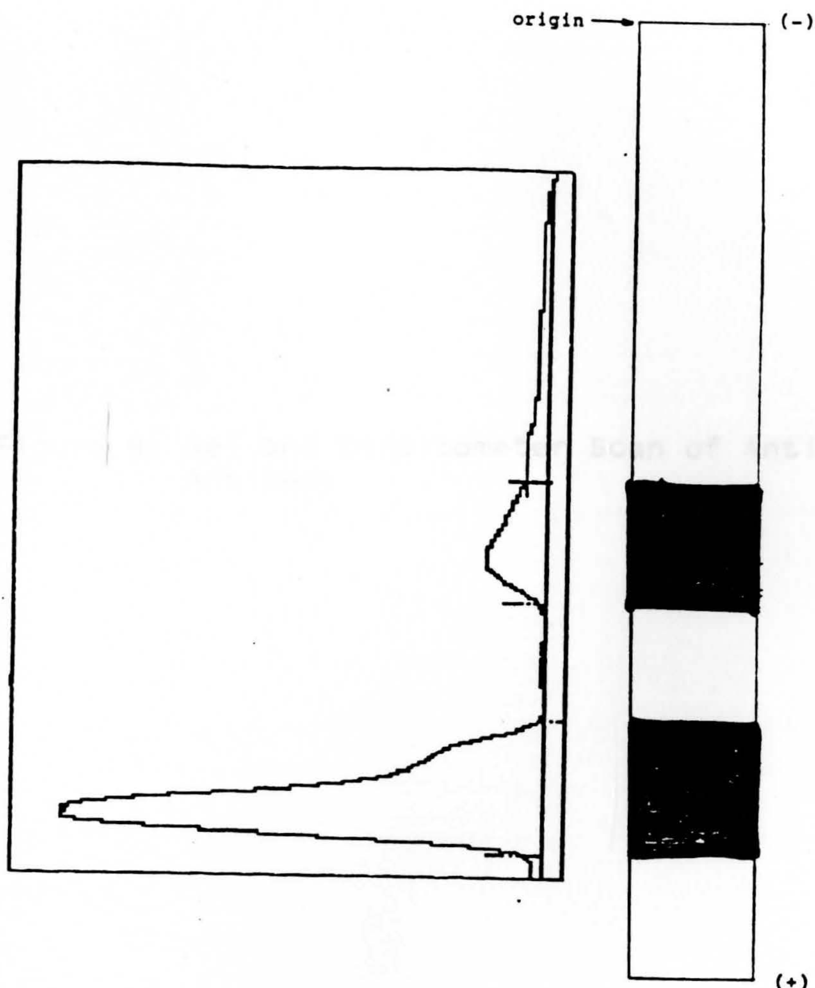


Figure 8: Gel and Densitometer Scan of Induced Membrane Fraction of <u>D. iridis</u> with Anti-A2 Antibody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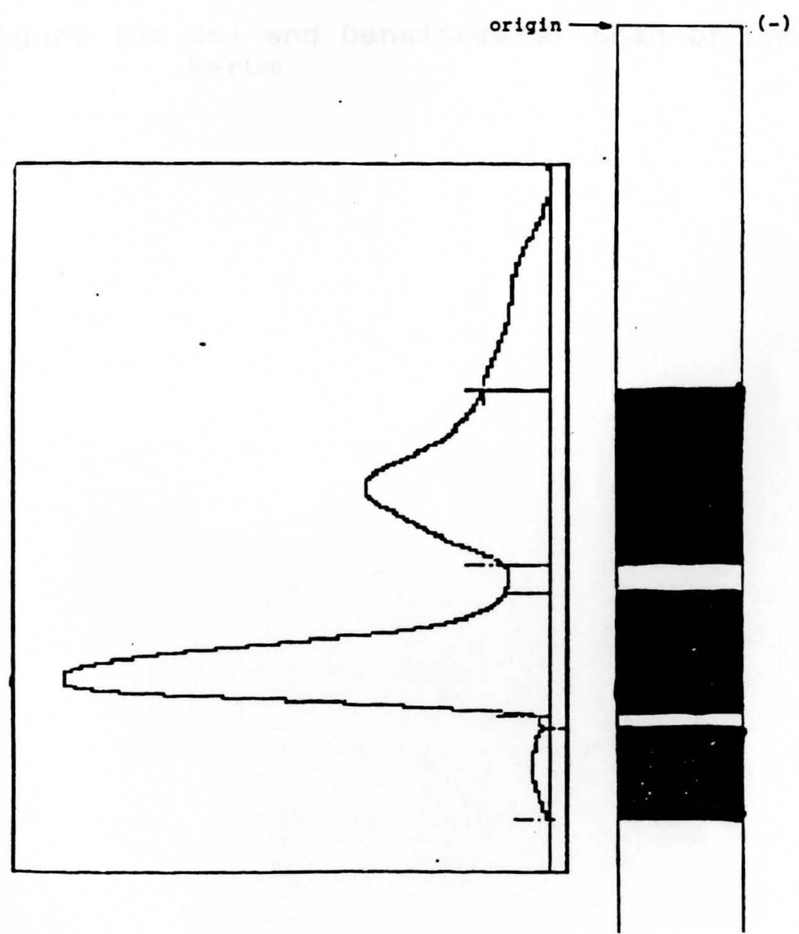
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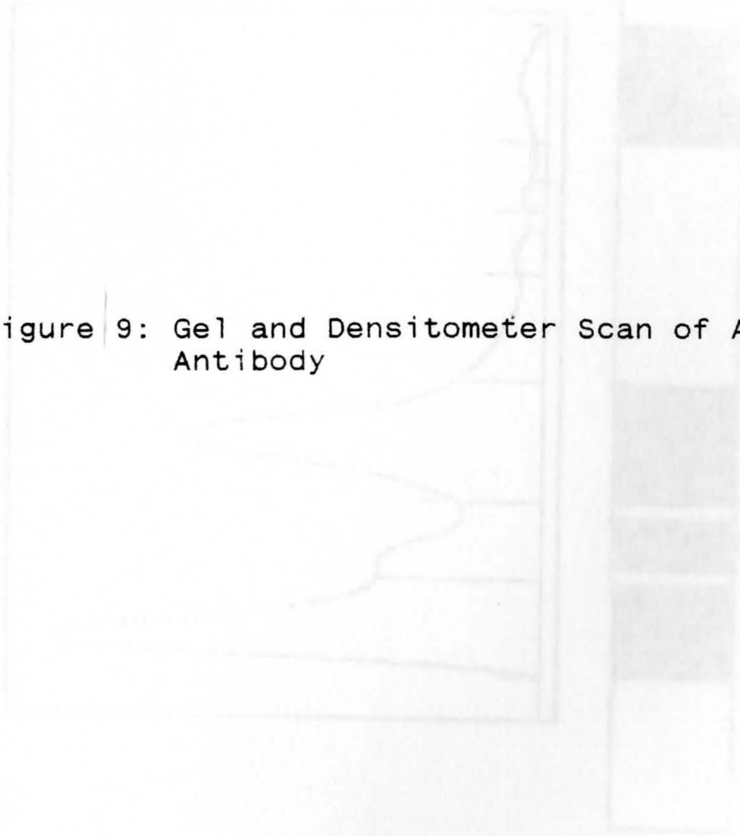
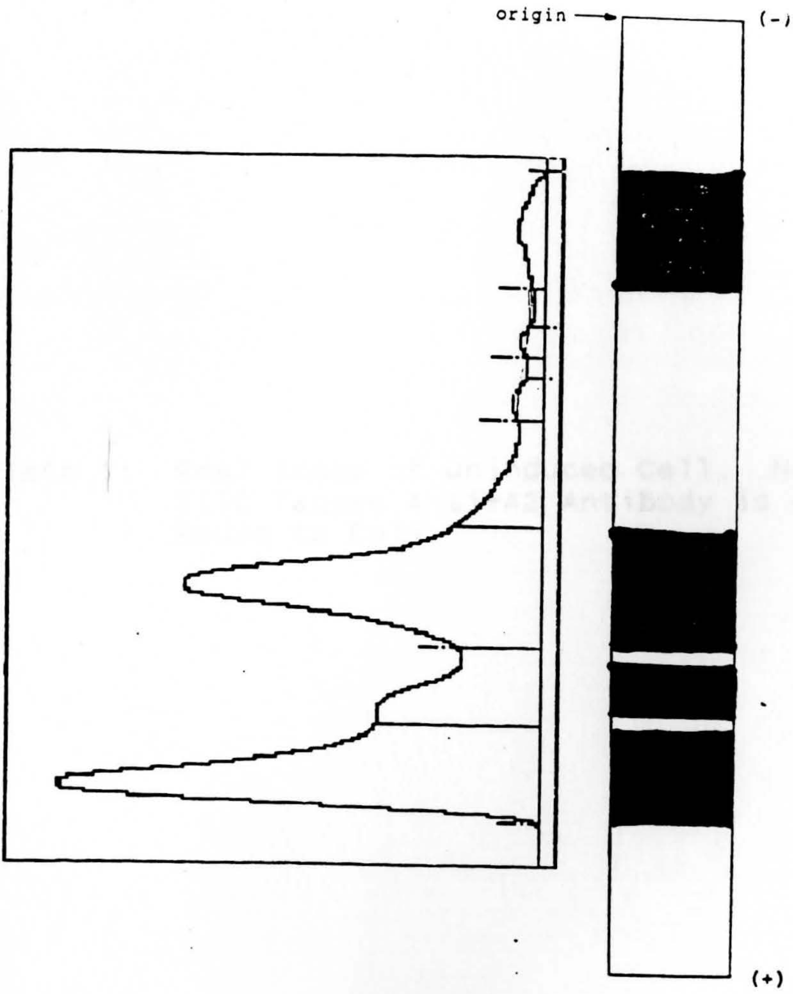


Figure 9: Gel and Densitometer Scan of Anti-A2 Antibody

Figure 10: Gel and Densitometer Scan of Control Serum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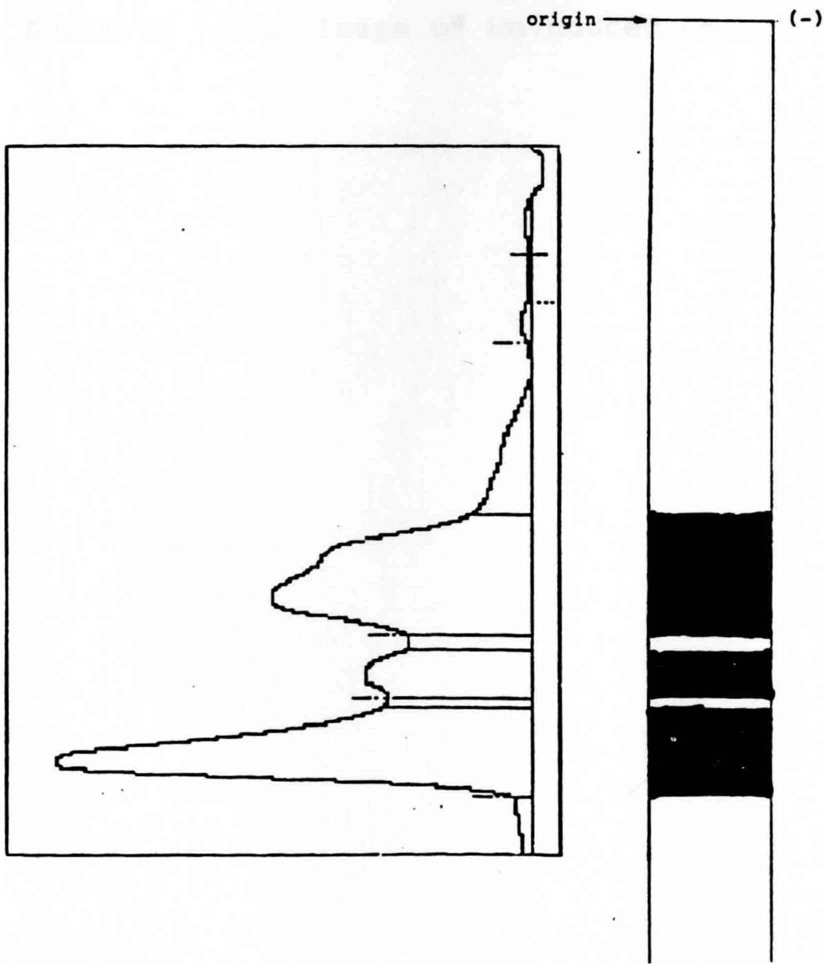
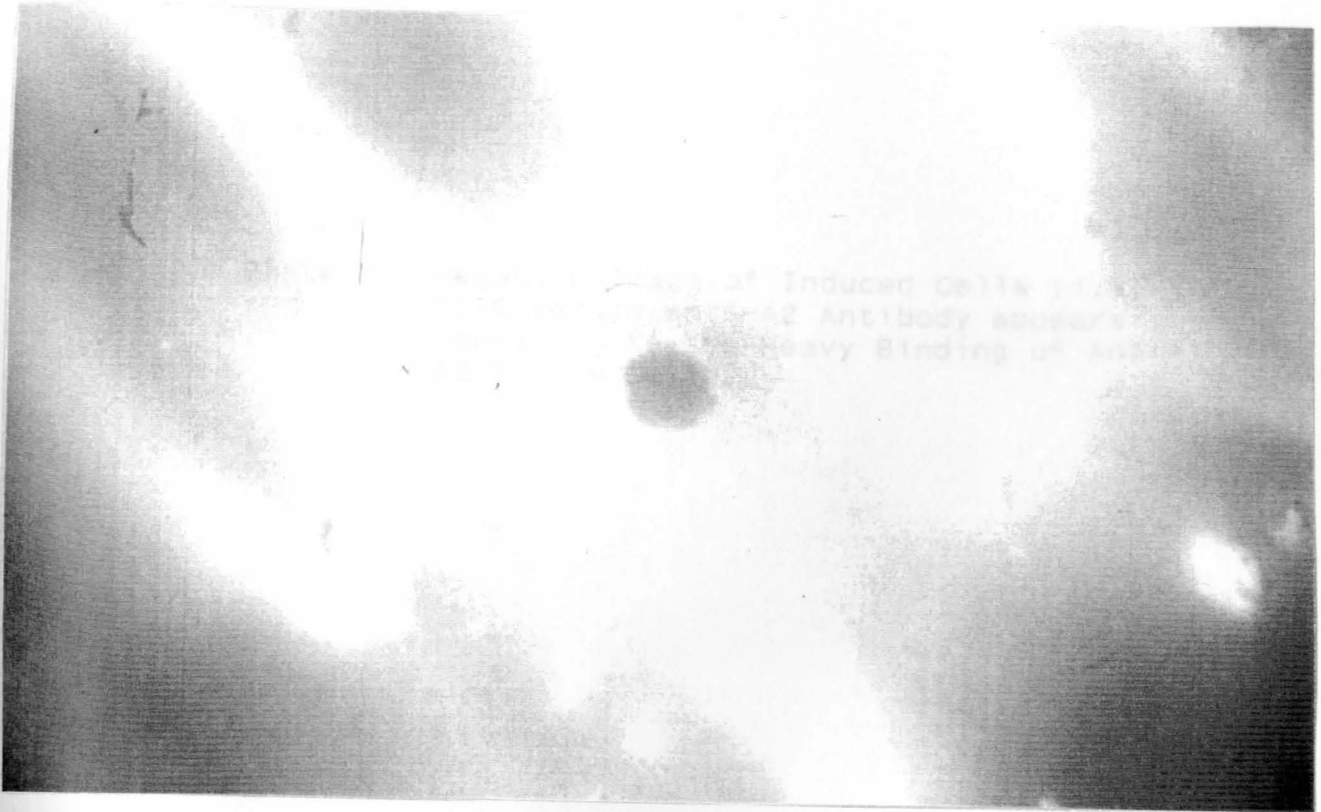

origin
(-)

Plate 1: Real Image of Uninduced Cell. Note how FITC Tagged Anti-A2 Antibody is not Bound to Cell.

Plate 2: Real Image of Uninduced Cell.



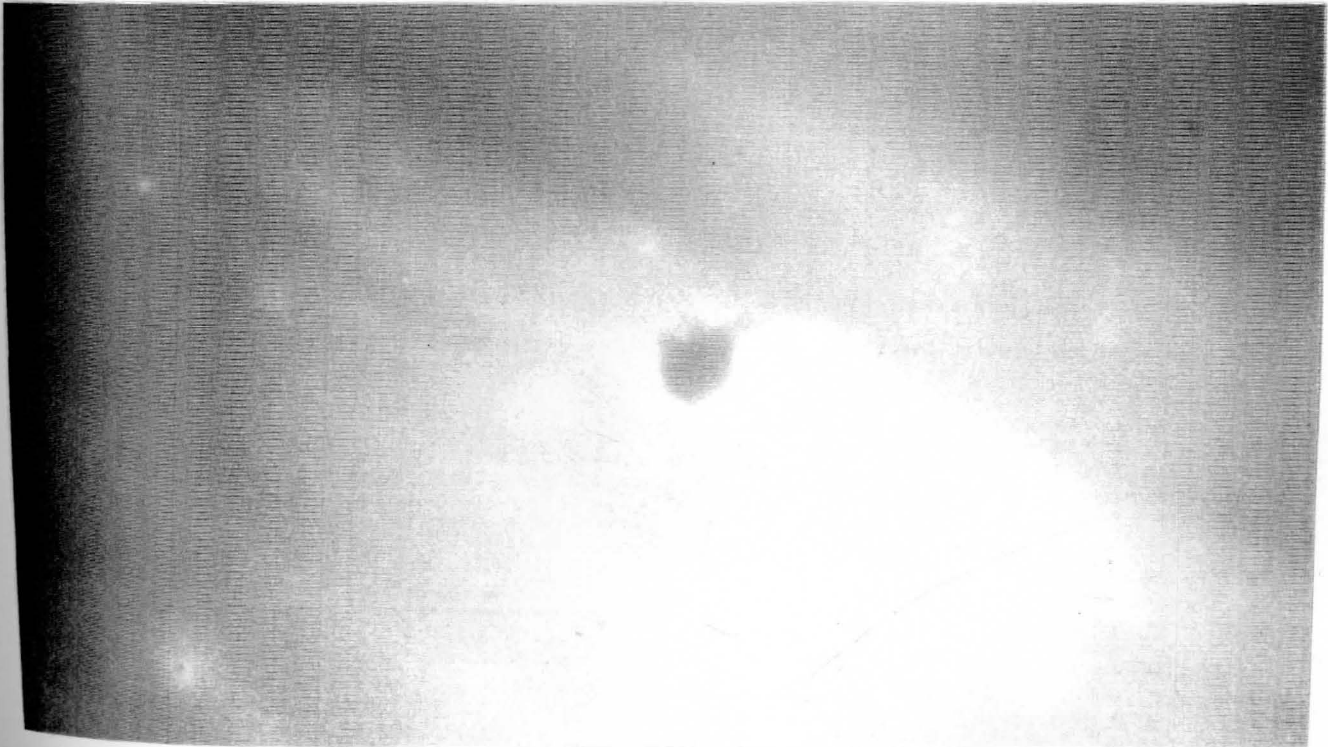
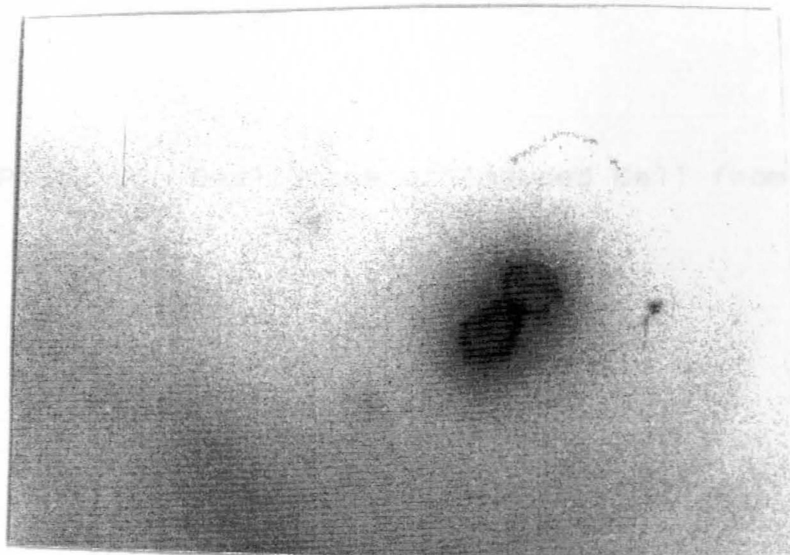
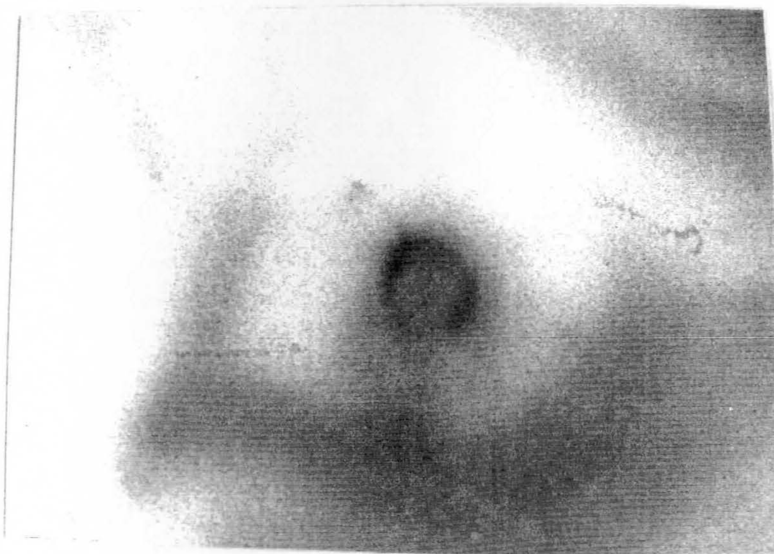


Plate 3: Negative Image of Induced Cells (i.e. FITC tagged Anti-A2 Antibody appears dark). Note the Heavy Binding of Anti-A2 to the Cells.

Plate 4: Negative Image of Induced Cell. Note Capping that Occurs at the Poles of the Cell.





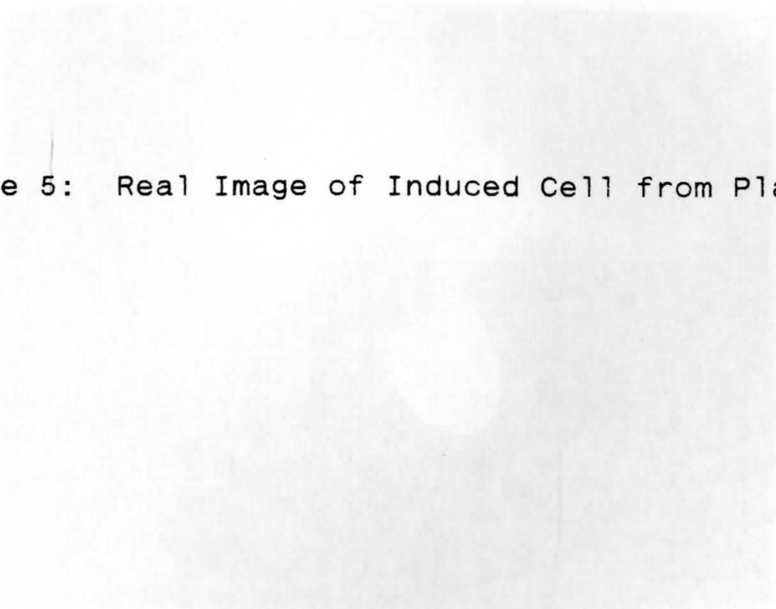


Plate 5: Real Image of Induced Cell from Plate 4.

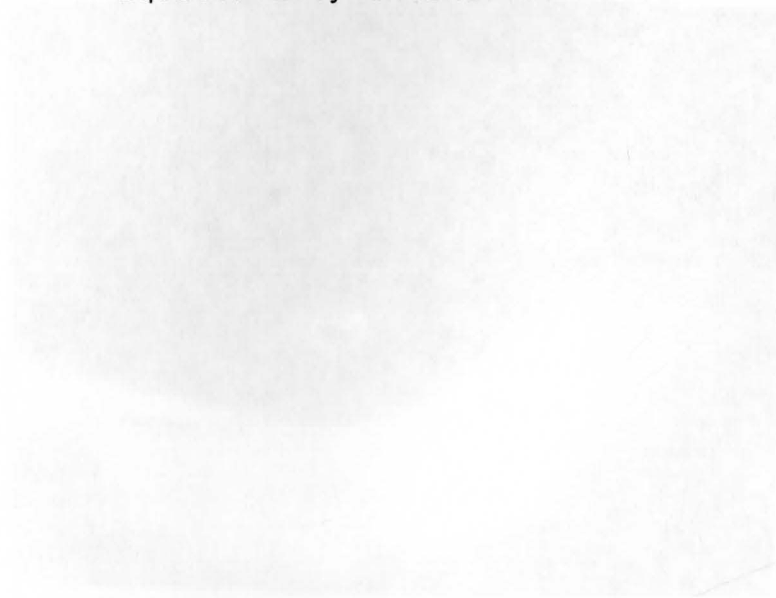
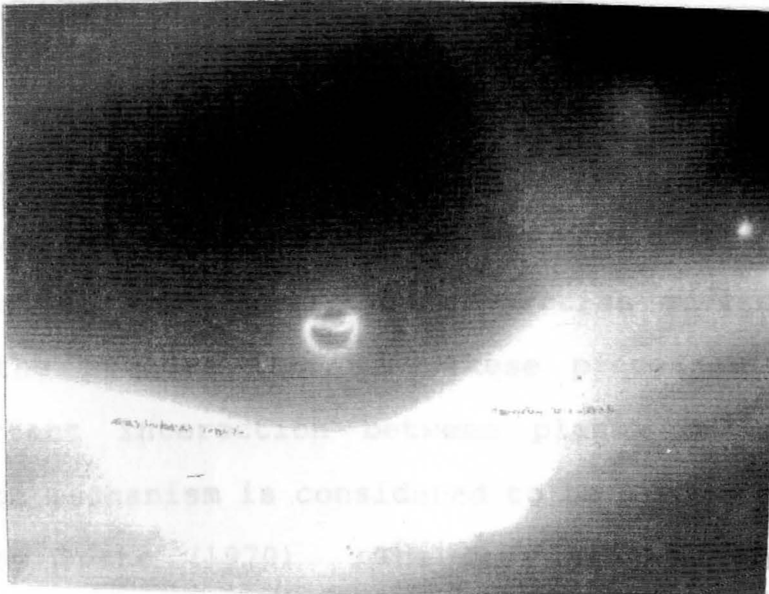


Plate 6: Real Image of Capping Occurring Equatorially on Induced Cell.



CHAPTER IV

Discussion

Rudolf Virchow extended the cell theory originally proposed by Schlieden and Schwann to include the premise that cells only arise from previously existing cells, ever since this extension of the cell theory, biologists have been fascinated by the dynamics of cellular interactions. Studies concerned with the unraveling the mystery of how one cell gives rise to another were contrasted with inversely related studies showing how two cells fused to generate one cell. The present study focuses on the latter of these two phenomena. Cell fusion occurs in cell types as diverse as human polykaryocytes (Hancox; 1965), cancerous cells and sex cells involved in zygote formation. The latter process can be aptly studied in the myxomycetes where haploid myxamoebae produce diploid cells by fusion. This study utilizes the myxamoebae in order to examine cellular fusion processes. Irrespective of the type of cell undergoing fusion, some ubiquitous properties govern the actual dynamics of the process itself. These processes involve the vitally important interaction between plasma membranes. The membrane fusion mechanism is considered to be a four stage process as proposed by Poste (1970). The four stages are: contact, induction, fusion and stabilization. Although most of Poste's theory arose from observations of polykaryocytes; ample similarities exist in all eukaryotic cell types to justify the use

of the four stage model to explain the fusion process occurring between haploid myxamoebae of D. iridis.

Of the four stages of cell fusion proposed, our studies concentrate on the protein constituents present in the plasma membranes that allow the actual cell fusion to occur. Previous studies have suggested the central role that proteins play in governing fusion between plasma membranes. Previous studies involving the induction of proteins and their roles in cellular fusion appear in the literature (Yemma and Stroh, 1991) The emphasis in the present study seeks to describe the actual physical process manifested in the fusion process, especially the role of mating receptors or factors present on the cell surface membrane. In a previous study it was found that proteases could attack membrane proteins and prevent the formation of plasmodia in D. iridis (Shipley and Ross, 1978). Because plasmodia usually only result after the successful fusion between two haploid myxamoebae of opposite mating type, it was concluded that fusion had been prevented due to the destruction of membrane fusion proteins. Membrane fusion proteins must also serve a recognition function to ensure fusion occurs with a suitable partner. The moieties that aid in the recognition are sugar residues linked to the membrane fusion proteins. Glycoproteins had been known to be distributed on the surface of myxomycetes for some time (Molday et al., 1976; Gillete et al., 1974) and experimental evidence supported the idea of glycoproteins participating in cell to cell adhesion. For example, an 80,000 MW glycoprotein of Dictyostelium discoideum was

shown to mediate cell to cell binding involved in aggregation (Siu, 1990). In <u>D. iridis</u> it was shown that glycoprotein interactions necessary for cellular fusion to occur could be blocked upon the addition of the lectin Concanavalin A (Yemma and Soltis, 1988). Because Concanavalin A preferentially binds oligosaccharides, this experiment dramatically demonstrated the necessity of glycoprotein interaction as a prerequisite to the resultant formation of diploid plasmodium from haploid myxamoebae.

As previously shown in Figure 1A both haploid and diploid phases are present in <u>D. iridis</u>. The morphological differences present between the haploid myxamoebae and the diploid plasmodium are striking. In addition, differences also reside within a given ploidy level, as seen by the interconvertability between the myxamoebae and the swarm cell flagellates (Figure 1A). Considering all the morphological differences seen in <u>D. iridis</u>, it can be assumed that many changes occur in the plasma membrane surrounding the differing cell types. Since lipoprotein, fluid membranes depend upon their intrinsic proteins to determine physiological interactions, it can be rationalized that the glycoproteins present in the plasma membrane must be in flux to accommodate the differences in cell morphology. Although morphological differences are obvious and can be visually observed, other more obscure but no less important changes occur in the glycoprotein constituency of the haploid myxamoebae to permit the fusion of their respective plasma membranes. Elucidation of the mechanism and constitutive components of this phenomenon has been the primary focus of our

laboratory for many years. Previous work in our laboratory has shown that upon reaching a critical cell density of 1x10 E+05 cells/ml haploid myxamoebae produce an inducer substance that changes the myxamoebae in such a manner that they become competent to recognize and fuse with cells of the opposite mating type (Yemma and Stroh, 1991). Yemma and Stroh (1991) also determined that haploid myxamoebae can induce themselves independent of any association with the opposite mating type. The inducer substance which appears to be a small organic acid is thought to function by altering (inducing) the glycoproteins present in the plasma membrane of the haploid myxamoebae (Yemma and Stroh, 1991). Thus upon addition of the inducer substance to uninduced cells (incompetent to fuse and mate) they transform into induced cells (competent to fuse and mate).

By determining which cell surface molecules were affected by the inducer molecule, it could be established which of these play a role in recognition and fusion. Before this study could commence it was necessary to obtain isolated plasma membrane fractions of induced and uninduced haploid myxamoebae. The technique developed by Restivo (1992) was utilized to collect the membrane fractions. Membrane fractions were used in conjunction with an antibody developed by injecting induced cells into rabbits. The antibody was produced against induced cells only and is referred to as Anti-A2. The A2 portion designates strain differences. Our study made use of the HON7A2 clone which can fuse compatibly with the PAN2-21A7B isolet used as the opposite compatible mating strain in this

study. Since HON7A2 is an A2 strain, Anti-A2 is able to recognize it and form the necessary antibody/fusion receptor complexes with it.

Early experiments were designed to determine if the Anti-A2 was able to prevent cellular fusion from occurring. As shown and described in the results section, Anti-A2 was indeed able to prevent fusion, when binding to the glycoproteins of the plasma membrane involved in the fusion reaction between haploid myxamoebae. This experiment was designed after the Yemma and Soltis' (1988) Concanavalin A blocking experiment which had initially determined that glycoproteins were the central participants in the fusion reaction. The blocking experiment utilized an immunolabeling technique to determine the apparent binding specificities of Anti-A2. In order to visualize binding, Anti-A2 was complexed to goat anti-rabbit IgG labeled with FITC. This fluorescent tag could then be easily visualized utilizing dark field fluorescent microscopy. The photographs showing that Anti-A2 preferentially binds to induced cells instead of uninduced cells are displayed in the results section (Plates 1-6). The preferential binding that Anti-A2 displayed was expected considering the origin of the Anti-A2. However, these findings suggesting that Anti-A2 bound to uninduced cells only to a slight extent was unexpected for this demonstrated that fusion factors were present on the plasma membrane in great numbers, perhaps all over the membrane (Plates 1 and 2). Our original expectations of some differences in Anti-A2 binding specificity between induced and

uninduced cells were thus confirmed, but the extent of how great these differences were was as explained surprising. Initially we anticipated many similarities to exist in the binding between induced and uninduced cells with Anti-A2. Comparing the photographs of the uninduced cells (Plates 1 and 2) with those of the induced cells (Plates 3-6) suggests that induced versus uninduced exhibits dramatic binding of antibody in the former. In fact, it appears that the entire membrane undergoes great change during the process of induction. The change is profound enough to allow only Anti-A2 to recognize the epitope presented by the induced plasma membrane. Evidently, great changes occur within the induced membrane itself as evidenced by the capping reaction evidently occurring in the induced cells (Plates 3 and 4). Also inferred from the photographic data is that induction clearly triggers widespread changes in the plasma membrane as evidenced by the HPLC data in the study of cell surface proteins. This phenomenon would be expected to accompany an event as vital to the species as sexual reproduction. Apparently, following induction the entire plasma membrane of the myxamoeba, undergoes a conformational change and presents a unique glycoprotein profile concerned almost exclusively with recognition and fusion events. This is consistent with studies done with D. discoideum that showed carbohydrate epitopes associated with glycoproteins changed significantly enough during various developmental stages so as not to be recognized by antibodies used previously (Crandall and Newell, 1989).

Following the outcome of the blocking and immunolabeling studies, it was necessary to study the fusion process in a more analytical manner. Our investigations required the use of an instrument able to detect interactions between Anti-A2 and the membrane glycoproteins. By employing the high resolving power of High Pressure Liquid Chromatography (HPLC) we were able to establish a standard protein or peak profile for both the induced and uninduced isolated plasma membrane preparations (Figure 1 and 2). In examining chromatographs, particular emphasis was placed on any changes occurring in retention time indicative of protein changes in the membrane studied. The reason being that two peaks with different retention times have been resolved on by high pressure liquid chromatography methods and can be considered separate entities. Our earliest experiments consisted of running the isolated membrane fractions alone to establish standard protein peak patterns unique to either the uninduced or induced membranes. Since all of our readings were made at a wavelength of 280nm, the resulting peaks represent the protein constituents of the isolated membranes. After numerous trials, it was determined that the uninduced membrane contained 3 unique protein peaks (Table 1) while the induced membrane contained only 2 unique peaks (Table 2). The finding of 3 uninduced peaks and 2 induced peaks and in agreement with the earlier studies of Restivo (1992). The retention times of all the peaks whether induced or uninduced in Restivo's study are nearly identical with those found in this study. It should be noted that other small peaks were occasionally observed on the

chromatographs but these peaks could not be consistently reproduced and are thought to be degradation elements of the larger more stable protein peaks. Only highly reproducible peaks are displayed in the chromatographs.

In comparing the uninduced chromatograph (Figure 1) and the induced chromatograph (Figure 2), the most striking observation is that of the presence of the additional peak in the uninduced sample. It was surprising to observe that the uninduced fraction had more peaks than the induced fraction. Our original presumption was that upon induction the haploid myxamoebae would generate membrane fusion proteins de novo and incorporate them into the plasma membrane. If this occurred then these new proteins would be observed as additional peaks in induced chromatographs when compared to uninduced ones. Apparently this is not the case. Fewer peaks are present following induction. Further analysis also reveals the presence of coeluting peaks existing between the uninduced and induced samples. The co-eluting peaks are the first peaks in each fraction namely, 3.10min and 3.08min peaks in the uninduced and induced respectively. These two peaks are believed to represent the protein constituents of the plasma membrane, shared by both groups. These co-eluting peaks were present in virtually every run of membrane fractions alone, suggesting that they have a high stability and possibly consist of proteins vital to the structure of the membrane and survival of the cell. Since this co-eluting peak is always present in both the induced and uninduced samples it is believed that this peak does not represent

the mating receptor. More likely this common peak is, as explained, structural in nature and is therefore shared in all membranes studied. However analysis of the remaining peaks does suggest that the mating factors are at this time present.

Previous work showing that Anti-A2 blocked fusion by binding the fusion proteins was incorporated into the design of experiments hoping to find evidence of Anti-A2/protein complexes. Complexes were detectable by noting that if upon addition of Anti-A2 to membrane fractions, retention times should change for the peaks of the membrane fractions, because complexed proteins should elute slower than would non-complexed proteins. In the uninduced fraction containing Anti-A2 (Figure 3) very little change occurs between this chromatograph and the chromatograph of the uninduced fraction alone. This result confirms the limited binding seen to have occurred between Anti-A2 and the uninduced cells in the immunolabeling experiments. However, when Anti-A2 was added to induced fractions dramatic changes in retention times were observed. The first peak of the induced chromatograph (Figure 2) had an increased retention time from 3.08 to 5.17 minutes upon addition of Anti-A2. The second peak in the induced fraction also showed an increase in retention time from 11.98 to 15.98 minutes upon Anti-A2 addition. This data suggests that a fundamental difference in antibody binding occurs between induced and uninduced membranes. Retention times have increased due the formation of Anti-A2/protein complexes. Although it was previously argued that the first peaks of the uninduced and induced chromatographs were

co-eluting and considered to be essentially comprised of the same proteins, one was bound by Anti-A2 while the other was not. The explanation for this appears to lie in the fact that, upon induction the entire membrane is altered. Included in this alteration are proteins always present in the membrane and not just proteins involved in recognition and fusion processes. It follows that if one considers that this co-eluting peak is possibly partially comprised of proteins involved in ion transport or some other physiologic process (since they are observed in both induced and uninduced fractions) along with other "in common" proteins that must be modified to aid in the fusion process in a more limited role to provide the necessary ionic and supportive conditions needed by the fusion proteins themselves. Proper conditions are a prerequisite to having fusion occur as stated by Poste (1970) in his Membrane Fusion Theory.

The second peak in the induced chromatograph is only found following induction and also complexes with Anti-A2 to a greater degree than the first induced peak as is evidenced by the retention time increase of 4 minutes as opposed to only 2 minutes upon addition of Anti-A2. Due to its strong ability to bind Anti-A2 and its appearance only following induction it is believed that this peak is centrally involved in the fusion process and is found in high numbers in the individual membranes. Strong binding of Anti-A2 to this second peak is evidenced by its nearly doubled increase in retention time when compared to the first induced peak. Strong binding had to have occurred in the blocking experiment to prevent

fusion, and this second induced peak definitely displays this property. The size of this second peak also aids in identification of its role in presenting the epitope recognized by Anti-A2 as shown in the immunolabeling study. Because of this ability to confer a unique epitope this further supports the idea that this peak is present only following induction. If this peak is indeed the membrane fusion receptor then the next question to be addressed is how this peak appears so quickly and in such great quantity. Yemma and Perry (1985) showed that induction was a very rapid process requiring anywhere from 20 minutes to one hour. This may not be a sufficient amount of time to produce a membrane fusion receptor de novo. Although this idea would have to still be confirmed perhaps by experiments involving the use of cycloheximide to block translation it does seem reasonable in light of the time constraint and large quantity (as evidenced by peak size) of the second induced peak present. The explanation for where this peak comes from may lie in further analysis of the uninduced peaks.

The two peaks present only in uninduced cells have retention times of 11.25 and 14.41 minutes. No uninduced peaks were found to form complexes with Anti-A2, while both peaks of the induced fractions did, thus suggesting that the entire membrane undergoes a conformational change upon induction. This data is verified by fluorescent antibody data. As previously shown by Crandall and Newell (1989) the glycoproteins are ever being modified in slime mold membranes throughout development. Apparently what is happening in our model is that instead of a de novo synthesis, we

have a conformational change in proteins already existing in the membrane. This change may even involve the possible hybridization of the unique uninduced proteins in forming the membrane fusion receptor present in the second induced peak. In comparing the uninduced (Figure 1) with the induced (Figure 2) chromatographs it can be clearly seen that the second induced peak elutes at a time between both the elution times of the unique uninduced peaks. This phenomenon suggests that hybridization may be occurring.

As stated in the results section due to its lower resolving power electrophoresis was used in this study to supplement our findings ascertained from the HPLC. Electrophoretic techniques were able to show that the uninduced and induced membranes were different initially (Figures 5 and 6) and also that Anti-A2 preferentially formed complexes with induced and not uninduced membrane proteins as shown by greater migratory pattern changes being present upon addition of Anti-A2 to induced and not uninduced fractions. Electrophoresis also generated an excellent means to ascertain the presence of the Anti-A2 in the serum. This is shown by the presence of an additional band of at 17.15 minutes in the Anti-A2 sample (Figure 9) which is absent in the control serum (Figure 10).

In conclusion, based on previous work in our laboratory and on the findings of other laboratories also working in this area we can reasonably assume that this second peak in the induced fractions is involved in the membrane fusion process. This peak, due to its

size, apparently provides Anti-A2 with a unique epitope and thus provides a unique recognition site for cells of the opposite mating type. Based on our work, further studies should concentrate on this membrane fusion receptor peak in order to determine its exact role in the fusion process. The next step besides determining whether or not this peak has a de novo origin via cycloheximide studies may be to use this peak to construct a monoclonal antibody (of course the mating factor would have to be isolated first) as opposed to the more general Anti-A2 raised against induced cells only however it must be remembered that membrane conformational changes occur and we do in fact have a fairly pure antibody preparation. If the monoclonal antibody can also block fusion then our results presented here suggesting the second induced peak contains the membrane fusion receptor will be confirmed and thus contribute to our knowledge and understanding of how two cells become one.

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