

**THE DETECTION OF GLYCOPROTEINS IN THE
PLASMA MEMBRANE OF *Dictyostelium discoideum***

Submitted in Partial Fulfillment of the Requirements for
Graduation with Honors to the Department of Biology and
Chemistry at Carroll College, Helena, Montana.

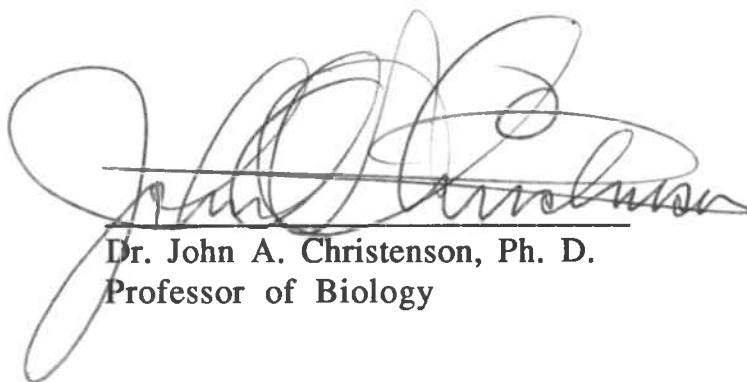
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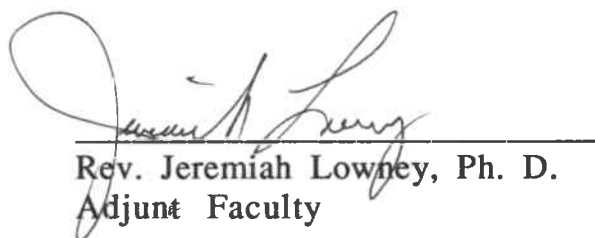


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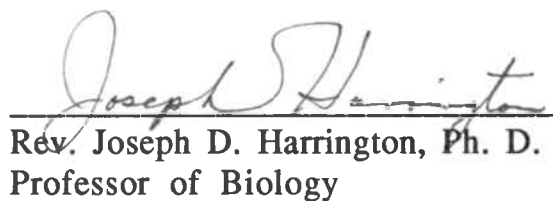
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ABSTRACT

A clone of *Dictyostelium discoideum* strain AX-3 was cultured up to its amoeboid stage. The glycoproteins in the plasma membrane of strain AX-3 were then isolated and purified. The proteins were fractionated by SDS polyacrylamide gel electrophoresis and then transferred to a nitrocellulose sheet by western blotting. The resulting nitrocellulose blot was used to detect glycoproteins with biotinylated concanavalin A and avidin-alkaline phosphatase. The results suggest there are mannose containing glycoproteins in the plasma membrane of strain AX-3.

TABLE OF CONTENTS

ACKNOWLEDGMENTS.....	i
ABSTRACT.....	ii
LIST OF ILLUSTRATIONS.....	iv
INTRODUCTION AND LITERATURE REVIEW.....	1
MATERIALS AND METHODS.....	4
Starting New <i>Dictyostelium discoideum</i> Clone.....	4
Storage of Slime Mold Stock in Silica Gel.....	5
Das/Henderson Membrane Preparation.....	6
Lowry Protein Assay.....	8
Polyacrylamide Gel Electrophoresis (PAGE).....	8
Western Blotting.....	8
Detection of Glycoprotein on Protein Blots.....	9
RESULTS.....	10
DISCUSSION AND CONCLUSION.....	11
LITERATURE CITED.....	13

LIST OF ILLUSTRATIONS

Fig. 1.	Development in <i>D. discoideum</i> , from aggregate stage to the mature fruiting body.	2
Fig. 2.	Sucrose gradient with isolated bands 1, 2 and 3.	7
Fig. 3.	Glycoprotein blots of <i>D. discoideum</i> plasma membranes with biotinylated lectins, avidin alkaline phosphatase and alpha-methyl mannoside.	10

INTRODUCTION AND LITERATURE REVIEW

The cellular slime molds are designated as the order Acrasiales within the phylum Myxomycophyta (Loomis, 1975). Several genera and many species have been discovered. However, the bulk of the current investigations has been carried out with *Dictyostelium discoideum*. Therefore much more is known of the morphological details and life cycle of this species than of any other.

D. discoideum, strain AX-3, is a cellular slime mold of interest because it can easily be grown in large quantity by axenic culturing and develops fruiting bodies rapidly and under a wide variety of environmental conditions. The life cycle is extremely important to investigators of this organism and is divided into four stages (Bonner and Eldredge, 1945). In the first stage (vegetative stage), the mold exists as single, uninucleated amoebae which phagocytize and undergo mitosis. The second stage (aggregation stage), which is initiated by depletion of the food supply, involves the aggregation of the single amoebae into a slug which, in the third stage (migration stage), migrate across the substrate secreting a slime sheath. In the fourth stage (culmination stage) of development, the slug rounds up and secretes a cellulose stalk containing vacuolated cells. On the apex of this stalk is the sorus, a mass of cells which have differentiated into spores. These spores, when released under favorable conditions, split open and the single, uninucleated amoebae emerge to begin a new life cycle.

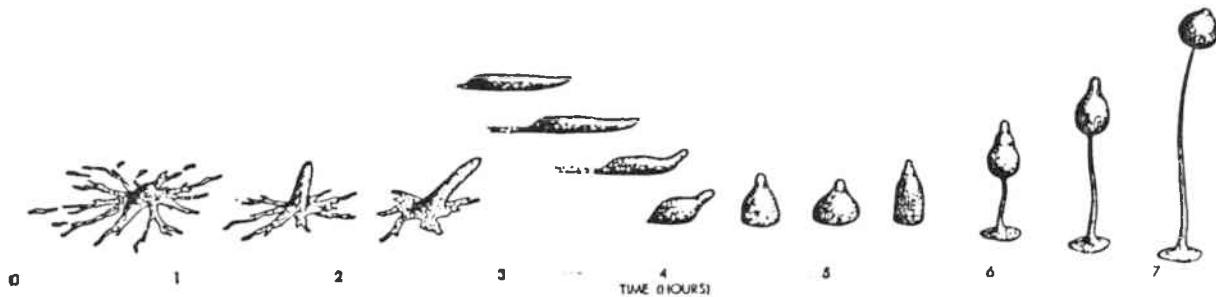


Fig. 1. Development in *D. discoideum*, from the aggregate stage to the mature fruiting body (from Bonner, 1967).

The purpose of this study is to characterize specific glycoproteins in the plasma membrane of *D. discoideum*. This is an attempt to identify molecules at the cell surface which are implicated in recognition and binding of food particles. The ultimate goal is to understand how signals from the environment or other cells are relayed through the cell membrane into the cell which ultimately brings about changes in the cell shape and movement. One possible cell surface molecule involved in the signaling process is the glycoprotein, and more specifically, the carbohydrate group attached to the glycoprotein. Glycoproteins are individual proteins that are complexed with a carbohydrate group. The carbohydrate units are located on the extracellular side of the plasma membrane. Identifying the carbohydrate units on each protein will categorize the glycoproteins into groups. Lectins, which are plant proteins with high affinity for specific sugar residues, are valuable probes for identifying the carbohydrate units on the glycoproteins (Stryer, 1988).

A study similar to my own was conducted by Brison and Carver. They used a variety of lectins including concanavilin A (con A) and wheat-germ agglutinin. The con A bound to internal and nonreducing terminals of alpha-mannosyl residues while the wheat-germ agglutinin bound to the terminal GlcNAc residue (Brisson and Carver, 1984). A study conducted by

Balding and Gold also used lectins to identify carbohydrates in the plasma membrane of *D. discoideum*. They used the lectin phytohemagglutinin, lectin A₄ from *Vicia villosa* and peanut lectin. They concluded that the phytohemagglutinin had an affinity for mannose and that both A₄ and peanut lectin had an affinity for GalNAc (Balding and Gold, 1975).

MATERIALS AND METHODS

A large quantity of *Dictyostelium discoideum*, strain AX-3 from the laboratory of Michael Brenner, Harvard University, was grown and retained in the amoeboid stage of its life cycle. This was necessary to keep the *D. discoideum* in a usable form and was accomplished by growing it in a liquid medium. The nutrients of this medium were kept abundant while the density of the cells was kept at a moderate level. This prevented the slime mold from finishing its growth cycle and kept it in the vegetative stage (Bonner, 1967). In this state the amoebae continued to proliferate at an exponential rate and could be easily collected and manipulated.

In order to inoculate *D. discoideum* spores in a liquid medium, it was first necessary to grow the slime mold to the fruiting body stage and then collect its spores. In preparation for growing the slime mold several sterile 2% bacto-agar plates were poured and one flask of LB broth was inoculated (50-ml in a 250-ml flask) using *Escherichia coli* B/r culture. The AX-3 spores stored at 4°C in silica gel, were brought to room temperature. The *E. coli* B/r culture was grown for 24 hr and then transferred to a 50-ml sterile conical centrifuge tube and pelleted. The supernatant was decanted except for approximately 0.75 ml. The pellet was then resuspended in the supernatant by vortexing. From the *E. coli* B/r suspension, 100 µl were pipetted onto three 2% agar plates and spread uniformly with a sterile glass pipette spreader. Approximately three to five silica gel particles were sprinkled onto the center of the agar plates. An additional 100 µl of the *E. coli* B/r suspension was then pipetted onto the silica gel particles.

Absorbing the moisture made the silica gel 'crackle' and break apart releasing the AX-3 spores. This violent reaction was necessary in order to spread the spores uniformly across the agar surface. The *E. coli* B/r suspension served as a food source for *D. discoideum* as well as the reagent to dissolve the silica gel. The plates were then stored in a humidified container in the dark at 20-22°C.

D. discoideum, strain NC-4 was also grown following the same protocol. However, NC-4 is not suitable for liquid media growth and thus was grown for observational purposes only. It usually takes 4-5 days before clearing of the bacterial lawn is visible (NC-4 grows 15-25% faster than AX-3). I monitored the plates daily and observed the steps of the life cycle microscopically.

By day 6 the AX-3 had matured to the fruiting body stage and was ready to be inoculated into medium. Fifteen mature fruiting bodies were carefully picked with a sterile loop and inoculated into HL-5 medium (5 ml in a 50-ml flask). To prevent bacterial growth, 10 µl of ampicillin were added to the HL-5 medium. The flask was then placed on a table shaker at 253 RPM. When the cells reached a density of 1×10^5 cells/ml, 1 ml of the suspension was transferred into HL-5 medium (750 ml in a 2-L flask) and spun at 253 RPM. The purpose of this transfer was to prevent a high density of cells from accumulating and possibly stimulating contact inhibition. The number of cells was determined with a haemocytometer and the generation time was calculated to be 9 hr. While the AX-3 was proliferating in the HL-5 medium, the remaining fruiting bodies were used to store slime mold spores in silica gel in order to keep a supply of the slime mold readily accessible.

Two-dram vials (7-8 ml) were filled with 3 g of silica gel. The vials were then dry sterilized. A 5% solution of milk (Carnation instant non-fat dried milk) was prepared in distilled water and autoclaved. The milk and the silica gel were cooled in an ice bath for 30 min. Considerable heat was produced when the gel was wetted. This heat could have killed the spores and was minimized by precooling the milk and the silica gel. Next the plates with the mature fruiting bodies were removed from the humidifier. The spores were harvested by banging the plates inverted on a working bench. Three plates were harvested into the same inverted lid. The spores were then resuspended in 2.4 ml of cold milk (0.8 ml per plate harvested). Then 0.4 ml of spore suspension were pipetted into each vial containing silica gel and was immediately capped and shaken vigorously for 5-10 sec. The vials were stored in Drierite at 0-5°C.

When the density of the AX-3 amoebae was roughly 1.0×10^7 cells/ml, the cells were harvested and prepared for the Das/Henderson membrane preparation. There were two main reasons why the Das/Henderson membrane preparation was used. First, the plasma membrane glycoproteins had to be excised from the membrane to be of use. Second, if the cells were lysed without being purified, cytoplasmic proteins would contaminate the preparation. The cells were harvested by centrifugation (GSA; 2,000 RPM; 2 min; 2°C) and then resuspend to $3-5 \times 10^7$ cells/ml in 200-250 ml of Prem Das' Lysis Buffer (PDLB) per bottle. The cells were then brought to room temperature and lysed using a 47 mm, 5 μ m nucleopore filter. This filtered out the intracellular organelles but allowed the membrane proteins and much of the plasma membrane to pass through. The 'filtrate' was collected in chilled bottles and pelleted (GSA; 6,000 RPM; 20 min; 2°C). The membranes were then washed twice with

cold PDLB (SS34; 18,000 RPM; 10 min; 2°C) and stored at 5°C. Next one sucrose gradient was prepared for every 1.5×10^9 cells by pipetting 500 μ l of a dense sucrose solution (1.8 M sucrose, 50 mM glycine, pH 8.5) into a SW 41 centrifuge tube. By using a gradient mixer, two other sucrose solutions were dispensed so that the heaviest solution (4.5 ml of 1.5 M sucrose, 50 mM glycine, pH 8.5) was on the bottom and the lightest solution (4.5 ml of 0.75 M sucrose, 50 mM glycine, pH 8.5) was on the top. The *D. discoideum* plasma membranes were then resuspended in PDLB by gentle homogenization. Two ml were then loaded onto each gradient and ultracentrifuged (SW 41; 29,000 RPM; 2°C; 18 hr). Three separate bands were identified (Fig. 2). Bands 1 and 2 were near the middle of the gradient and close to overlapping while band 3 was located just above band 2. Bands 1 and 2 were collected as one sample and band 3 was

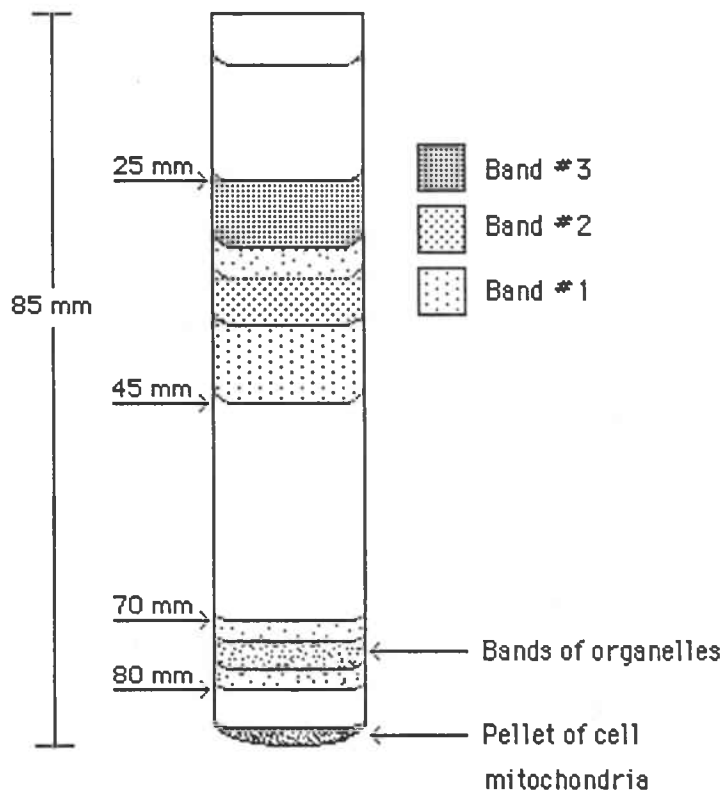


Fig. 2. Sucrose gradient with isolated bands 1, 2 and 3.

collected separately. Both samples were washed twice in cold 20 mM NaPO₄ (SS34; 18,000 RPM; 30 min; 2°C), inoculated with 0.02% NaN₃ (anti-bacterial agent) and then stored on ice. A small quantity of the membranes was fast frozen and stored at -80°C.

A Lowry Protein Assay was performed on bands 1 and 2 to determine their protein concentration. Band 3 was not assayed because it was not as pure and was more diffuse than bands 1 and 2. The wavelength of the spectrophotometer was set at 750 nm. Following the Lowry assay, the protein concentration was determined to be 1.4 µg/µl for bands 1 and 2 and 14 µg/µl for the crude membranes.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to estimate the protein subunit molecular weights and to determine the subunit compositions of the purified proteins through a western blot. The SDS-PAGE rather than a standard PAGE was used to impose a uniform charge on all the proteins in the sample mixture (Garfin, 1990). Two 8%-18% gradient gels were prepared. Forty µg of band 1 and 2 and of the crude membranes were sufficient to deposit a detectable amount of protein in the gel. The samples were electrophoresed at 90 v or 26 ma for 3.5 hr then submerged in transfer buffer in preparation for western blotting.

Western blotting is a useful technique because it transfers the proteins from the SDS-PAGE gel to a nitrocellulose sheet where they can be more easily manipulated. The gel was transferred for 60 min at 150 ma. When the blot was completed, the cartridge was disassembled and the blot was placed in blocking buffer.

The blot was then cut into three pieces in preparation for the detection of glycoproteins on the blots with biotinylated lectins. The blot strips were

placed into three individual bags. In bag A 80 μ l of biotin con A were added to 16 ml of lectin stain solution. In bag B 50 μ l of alpha-methylmannoside were added to 10 ml of lectin stain solution and to bag C 10 μ l of straight lectin stain solution were added. The bags were then sealed and incubated for 1 hr. Following the incubation period, the blots were removed from the bags and washed in washing solution I (50 ml 10X TBS and 5 ml 10% TX-100 in 500 ml ddH₂O) for 4x10 min. Upon the completion of the wash, the blots were rebagged and incubated in avidin alkaline phosphatase for 1 hr. The blots were then rewashed in washing solution II (9.53 g Na₂B₄O₇·10H₂O; 75 ml 1 M NaCl and 2.5 ml 10% TX-100 in 500 ml ddH₂O) for 2x10 min and then equilibrated by adding AP 9.5 (2x10 min). Next the color development step was initiated by adding the blots to 222 μ l of 5% BCIP in 66 ml of warmed AP 9.5 NBT solution. The blots were incubated for 20 min and then rinsed in stopping solution and placed on filter paper to dry.

RESULTS

Blots	Biotin Con A	Avidin Alk.- Phosphatase	Alpha-Methyl- Mannoside	Color Indication
A	+	+	-	Positive
B	+	+	+	Negative
C	-	+	-	Negative

Fig. 3. Glycoprotein blots of *D. discoideum* plasma membranes with biotinylated lectins, avidin-alkaline phosphatase and alpha-methyl mannoside. Blot C = control.

Blot A (Fig. 3) was exposed to biotin con A and avidin alkaline phosphatase but was not exposed to alpha-methyl mannoside. The result was an indication of color on the blot. Blot B was incubated in the presence of all three reagents. However, no color was indicated on the blot. Blot C was not exposed to biotin con A or alpha-methyl mannoside, but was incubated in avidin-alkaline phosphatase. The result was again an absence of color from the blot.

DISCUSSION AND CONCLUSION

Blot A (Fig. 3) was incubated in the presence of biotin con A and avidin alkaline phosphatase but without alpha-methyl mannoside. The result was that biotin con A could bind to the carbohydrate on the protein and the avidin alk. phos. would likewise bind to the biotin con A. Thus on blot A the color reagents could be seen. Blot B was incubated in the presence of all three reagents. As a result the alpha-methyl mannoside attached to the biotin con A which in turn bound to the avidin alk. phos. Thus no color reagents could be seen because the alpha-methyl mannoside attached to the biotin con A, preventing it from attaching to any binding sites on the carbohydrate. This was done to show that biotin con A and not some other substance during the incubation was binding to the glycoprotein. Blot C lacked biotin con A and alpha-methyl mannoside, but had avidin-alk. phos. Therefore no color reagent could be seen, because the avidin-alk. phos. has no binding site to attach and will remain in solution. This blot was to show that avidin-alk. phos. was not binding to the blot in place of con A.

As a result of all three blots, it was obvious that biotin con A was the reagent attaching to the blot. This demonstrates that at least some of the glycoproteins in the plasma membrane of *D. discoideum* can be bound to with a biotinylated lectin such as con A. These results correspond with those of Brisson and Carver (Brisson and Carver, 1984).

The protocol was sufficient to determine the presence of glycoprotein in the *D. discoideum* membrane. However, it did not give suitable data for obtaining the quantity of glycoprotein. The quantity would be useful for creating ratios of the amount of different glycoproteins in the plasma

membrane. The method used to determine the presence of glycoproteins was probably not sensitive enough to detect all bands on the blot because of the minute amounts of carbohydrate present.

In further studies, the same procedure could be applied with other biotinylated lectins until the majority of the glycoproteins had been characterized. With additional research it may be possible to understand how signals from the environment or other cells are relayed through the cell membrane into the cell bringing about changes in cell shape and movement.

D. discoideum must actively ingest food particles to obtain nutrition. This activity is called phagocytosis and it involves several steps including: 1) recognizing a food particle, 2) binding the particle, and 3) engulfing the particle. This process is fundamental to many cell types including ones in the mammalian immune system such as macrophages and neutrophils. If we could better understand how signals from the environment bring about phagocytosis, then it may be possible to control phagocytosis in macrophages and neutrophils. This would enhance the immune system and lead to numerous other medical advances.

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