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DIFFERENCES ASSOCIATED WITH MATING
TYPE ALLELES IN MYXOMYCETES

BY

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B.S., University of Central Florida, 1977

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Biological Sciences
in the Graduate Studies Program of the College of Natural
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1982

ABSTRACT

Differences associated with the mating type alleles of Didymium iridis and Physarum polycephalum were examined with fluorescent antibody and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Heteroabsorption of each anti-myxamoebal serum followed by testing serum activity using immunofluorescence showed there is no strain-specific activity in any of the anti-myxamoebal sera, but the sera was shown, to be genera specific. Intergeneric differences and similarities were shown in the electrophoretic patterns of the myxamoebal protein extracts from P. polycephalum, D. iridis, and Dictyostelium discoideum when compared. Intraspecific differences were noted in D. iridis.

ACKNOWLEDGEMENTS

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Lastly I want to thank my wife Sherri for her unending support and inspiration and for time spent typing this thesis.

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INTRODUCTION

The Myxomycetes or plasmodial slime molds constitute a natural group of eukaryotic organisms encompassing about 500 known species (Collins, 1979). Anton de Bary, one of the first investigators to become interested in these organisms, proposed the name Mycetozoa for a group which included the plasmodial slime molds and the cellular slime molds. Although de Bary (1860) recognized that Mycetozoa have fungal characteristics, he placed the major emphasis on the group's animal similarities. Link (1833) first applied the name Myxomycetes to the plasmodial slime molds. This places the major emphasis on their fungal similarities.

The reason for the controversy of whether to place the Myxomycetes in the plant or animal kingdom is based on different stages of their life cycle. The botanists argued that the Myxomycetes exhibit aerial fruitification, have a stipitate habit of higher forms, and attach themselves to some fixed base. The zoologists argued that if hypae are a morphological test of a fungus, then the Myxomycetes are not fungal, because they have no hypae. There are, however, certain parasitic fungi, Chytridiaceae, that lack hypae. (See Hawker, 1952; Alexopoulos, 1963, for reviews.)

The controversy remains unresolved, but G.W. Martin

(1940) in one of the major taxonomic works dealing with the Myxomycetes, described his Division Fungi and recognized the Myxomycetes as a class along with the Phycomycetes, Ascomycetes, and Basidiomycetes. In 1960 Martin revised his classification dividing his Division Mycota (Fungi) into two Subdivisions: Myxomycotina and Eumycotina. The Myxomycotina include the single class Myxomycetes with two subclasses Ceratiomycomycetidae (Exosporeae) and Myxogastromycetidae (Myxogastres), the former with the single order Ceratiomyxales, the latter with five orders: Physarales, Stemonitales, Echinosteliales, Trichiales, and Liceales (Martin, 1960; Alexopoulos, 1963). Other investigators have chosen to follow de Bary's conclusion that the Mycetozoa should not be placed in the plant kingdom and have placed them in the phylum protozoa. (See Olive, 1970, for a review.)

The Myxomycetes (plasmodial or acellular slime molds) have often been grouped with the Acrasiales (cellular slime molds) (Alexopoulos, 1952), the Plasmodiophorales (Ellison, 1945), and the Protosteliida (Olive and Stoianovitch, 1966). In the Myxomycetes there is a formation of a true multinucleated plasmodium. Flagellated swarm cells are typically a part of the life cycle. Fusion of cells behaving as gametes appears to be a prerequisite for the completion of the life cycle of most species (Martin, 1940; Alexopoulos, 1963). In the Plasmodiophorales, the presence of a

zoosporangia, the absence of a fruiting body, and the peculiar type of nuclear division they exhibit defies a close relationship (Karling, 1942). The strongest argument for the relationship of the Myxomycetes to the Plasmodiophorales is furnished by the evidence that the swarm cells of both groups are heterokont; possessing two anterior flagella of the whiplash or modified whiplash type (Ellison, 1945; Bessey, 1950; Alexopoulos, 1952). In the Acrasiales (cellular slime molds), there was no formation of a true multinucleate plasmodium, no fusion of cells behaving as gametes, and no flagellate swarm cell. The strongest argument for the relationship of the Myxomycetes to the Acrasiales is that both possess fruiting body structures and myxamoebae as part of their life cycle (Bonner, 1959). In the Proto-steliida, the fruiting body most often consists of a single spore, the trophic stage varies from uninucleate amoeboid cells to reticulate plasmodia, and flagellated cells similar to the Myxomycetes are present in some genera. Sexuality has not been demonstrated in any of the genera (Olive and Stoianovitch, 1966).

Although much work has been done on the life cycle of the Myxomycetes, only a few species have been done in detail. The generalized life cycle for the Myxomycetes is usually based on Physarum polycephalum Schw. (Howard, 1931). Not all life cycles are alike and the cycles may vary from species to species (Koevenig, 1961, 1964; Kerr,

1967; Aldrich, 1969; Carilile, 1974). The general life cycle of the Myxomycetes is as follows (Fig.1). The spore germinates giving rise to swarm cells or myxamoebae. These fuse in pairs to form zygotes. A zygote may grow by itself or fuse with other zygotes to form and develop into a multinucleate mass of protoplasm called a plasmodium. The plasmodium matures, and as certain environmental conditions prevail it becomes converted into fruiting bodies which bear the spores. There are two resting stages in the life cycle. The swarm cells or myxamoebae may encyst and the plasmodium may form a sclerotium, which is composed of macrocysts. These resting stages are not needed for the completion of the life cycle but occur simply as a result of unfavorable conditions (Howard, 1931; Ross, 1957; Koevening, 1961).

Although the sequence of stages in the cycle is generally the same from one species to the next, there are several different nuclear cycles (Fig. 2). Each of these may be viewed as a separate and alternative reproductive system. The system is subdivided into two major categories, heterothallic and nonheterothallic. Organisms placed in the first group possess mating types and are sexual, whereas those categorized as nonheterothallic are not known to possess mating types and may be either sexual (homothallic), or nonsexual (apomictic) (Collins, 1979).

The main vegetative stage of the true slime mold is the plasmodium. It is generally believed that a fusion of

Fig. 1. Life cycle of Didymium iridis .

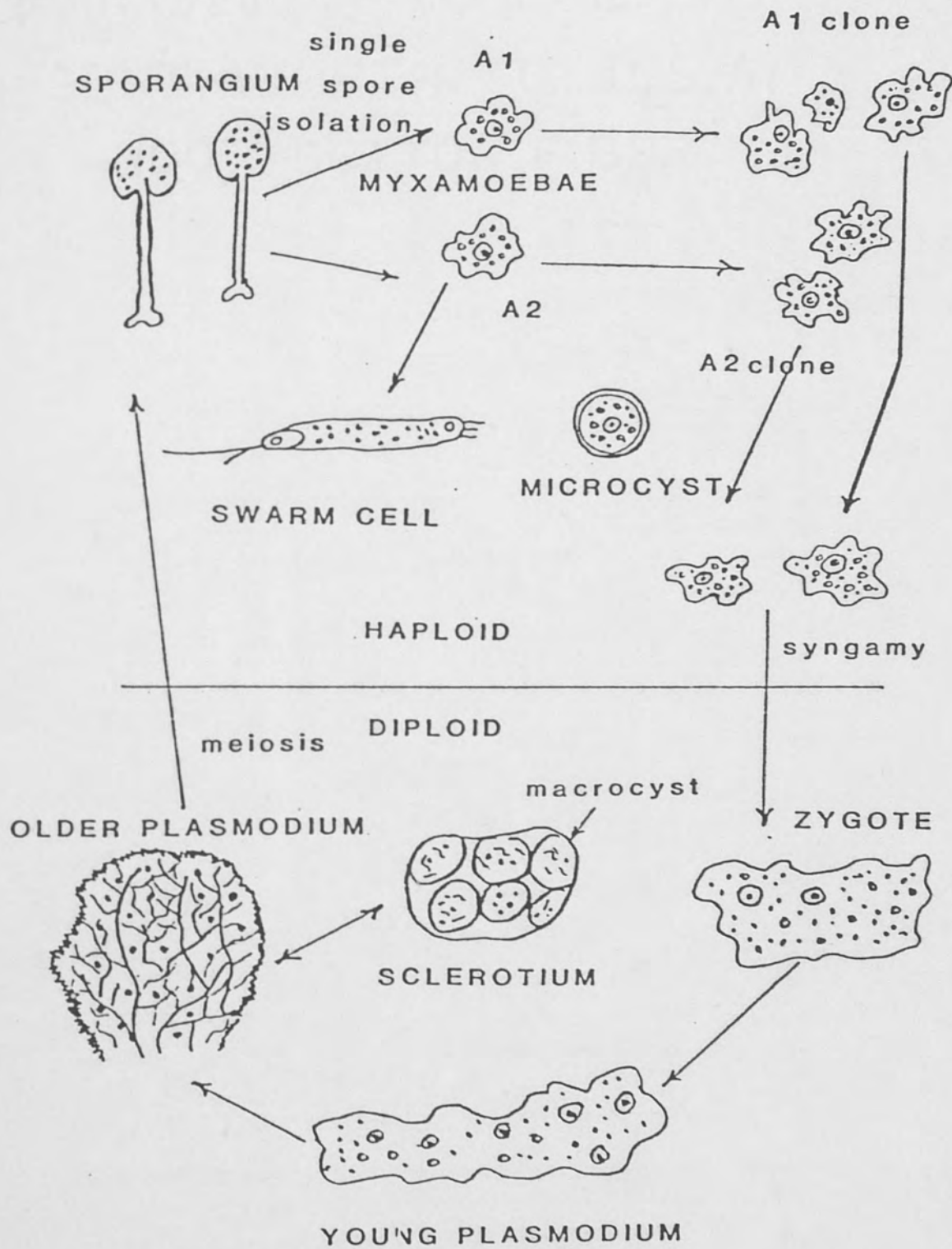
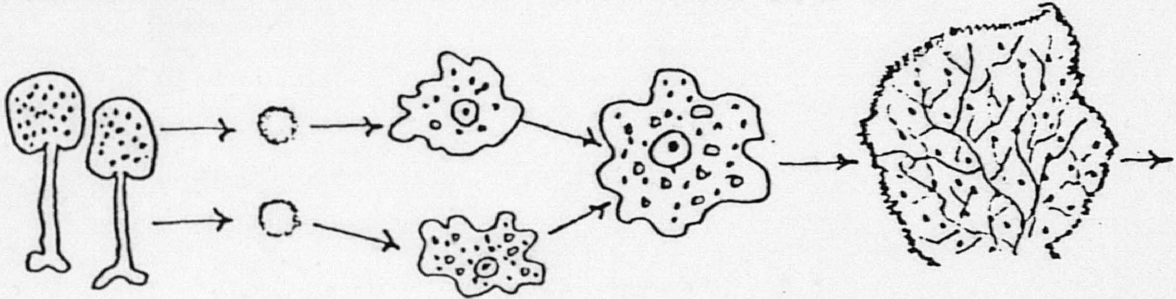


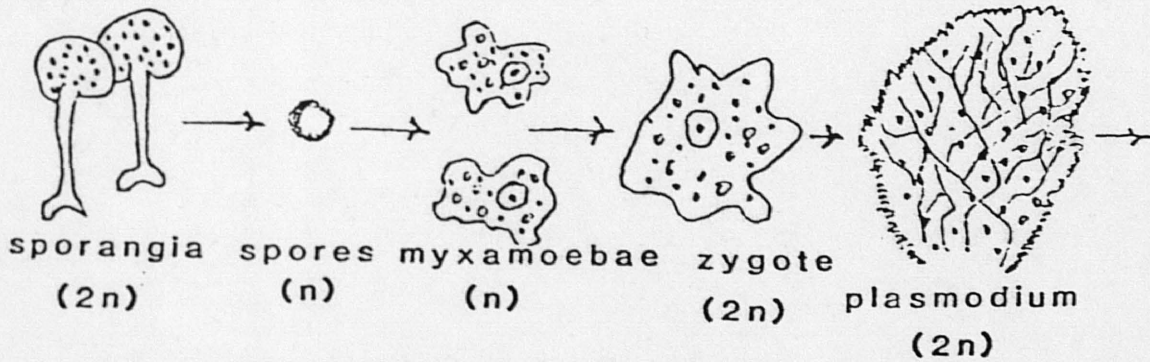
Fig. 2. Nuclear cycles of Didymium iridis.

SEXUAL CYCLES

HETEROTHALLIC (isolates with mating types)

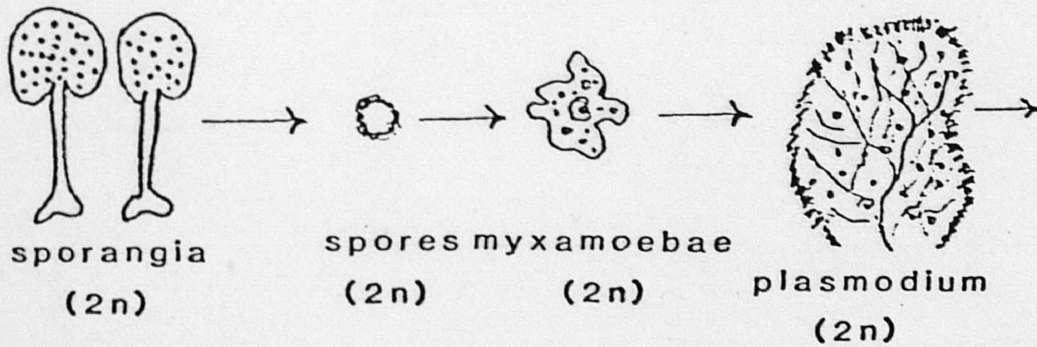


HOMOTHALLIC (isolates without mating types)



NONSEXUAL CYCLES

APOMICTIC (isolates diploid throughout)



amoeboid or flagellated protoplasts is a prerequisite for its formation. Plasmogamy, the fusion of two or more protoplasts, was first described by Cienkowski (1863) between myxamoebae. Jahn (1911), Skupiński (1926), Gilbert (1928), and Schunemann (1930), also reported fusion between myxamoebae. Wilson and Cadmann (1928) found that Reticularia lycoperdon Bull. plasmogamy occurred between swarm cells and not myxamoebae. Koevenig (1964) reported plasmogamy between myxamoebae only in Didymium iridis (Ditmar) Fries and Fuligo cinerea (Schw.) Morgan.

Abe (1933), working with Fuligo septia, reported that one swarm cell got smaller as the other got larger, eventually flowing into the other. Wilson and Cadmann (1928) described the fusion of swarm cells and observed that fusion always occurred at the posterior ends as did the ingestion of particles. Ross (1957), in a systematic study, found plasmogamy in nineteen species of Myxomycetes. Ross found that plasmogamy occurred either between swarm cells or between myxamoebae and was dependant on the conditions of the cells at the time of fusion. The condition of the cell was only temporary and could be altered by a change in environmental conditions. However, it was shown that a plasmodium could develop from a single protoplast or a clone that was derived from spores without the benefit of crossing (Kerr, 1967; Henney, 1967). This apparently is a very rare occurrence which happens only in unusual circumstances. Dee

(1960), working with Physarum polycephalum Wisconsin 1 (Wis 1) obtained amoebal strains by cloning and classified these strains into two mating types. These alleles were later designated mt_1 and mt_2 . Plasmodia were rarely produced in a clone from a single amoeba, but when amoebae of different mating type were mixed, plasmodia were readily obtained. Collins (1961), working with D. iridis, also reported mating types (heterothallism); this work was later expanded to genetic analysis of three different geographical isolates, and as a result it was shown that each heterothallic isolate typically carries a pair of genes which control mating and segregate during meiosis as two alleles at one gene locus (Collins, 1963; Collins and Ling, 1964). When the clonal amoebal cultures are crossed in all pairwise combinations, the clones can be assigned to mating types on the basis of whether plasmodia are produced in the cross (Dee, 1962; Collins, 1963). Dee (1960) and Collins (1961) were not the first to report mating strains (Skupiński, 1926; Cayley, 1929; Martin, 1940), but they clearly established heterothallism in the Myxomycetes, i.e., require the fusion of two compatible gametes of opposite mating types for the formation of a zygote and of a plasmodium. Dee concluded that the production of plasmodia must involve fusion between amoebae of different mating types (plasmogamy) and that fusion of nuclei would yield a diploid state. The haploid state would be restored by meiosis before or during sporulation. In

support of Dee's conclusions, electron microscopy firmly established that meiosis occurs during spore maturation in P. polycephalum (Aldrich, 1967) and in other Myxomycetes (Aldrich and Mims, 1970; Aldrich and Carroll, 1971). Dee's conclusions were further supported by DNA determination in nuclei (Mohberg and Rusch, 1971) and by chromosome counts, which showed amoebae of Wis 1 isolate were haploid and plasmodia were diploid (Mohberg et al., 1973). Other mating types were found in P. polycephalum which were allelic to the Wis 1 strain and were designated mt₃...mt₁₄. Many of the amoebal strains of P. polycephalum are heterothallic, with mating controlled by multiple alleles at a single locus (Collins, 1961; Collins, 1975; Collins and Tang, 1977). The first exception to the general rule of heterothallic mating as a prerequisite for transition was the isolation of Colonia (CL), an amoebal strain which forms plasmodia clonally (Wheals, 1973; Cooke and Dee, 1975). Its phenotype was found to be associated with an allele of the mt locus, designated mth for "mating type homothallic." To be truly homothallic, CL plasmodia would have to undergo a reduction to regain the haploid level of DNA. Present genetic evidence indicates that the CL strain when crossed with heterothallic strains also yields recombinant progeny and therefore undergoes nuclear fusion and meiosis. Microdensitometric measurements of nuclear DNA content (Mohberg et al., 1973) indicated that CL plasmodia have the same G₂ nuclear

DNA content as CL amoebae. This excludes the possibility of nuclear fusion and homothallism. An alternative hypothesis would be that CL is apomictic, where mating of myxamoebae is not necessary to yield plasmodia. In P. polycephalum sexual recombination is ordinarily an obligatory step in the life cycle. It is part of the process linking the vegetatively growing, haploid, amoebal form; and the multinucleate, diploid, plasmodial form.

It is generally thought that the informational exchange crucial to such processes as tissue morphogenesis, development, and sexual union of gametes is mediated by contact between cell surfaces. There are two theories to explain the exchange. One proposes that it is due to differential adhesiveness between various cell surfaces as a whole (for instance due to electrostatic charge or other collective molecular effects of conformation) (Poste and Allison, 1971; Ross et al., 1973). The other proposes that it involves the interaction of a small number of specific cell surface components (Carlile, 1976; Gerisch, 1976). The first theory is difficult to test experimentally. Abe (1933) studied mating Myxomycete cells and found that there was a difference in the oxidation - reduction potential, and in the resistance against cation (Cu) and anion (CN-) of mating types. Kambly (1939) repeated Abe's experiments and found no difference between the fusing cells. Kerr and Sussman (1958) found that a 2% (W/V) glucose or a 0.2% (W/V) brucine

solution prolonged the myxamoebal stage of Didyium nigripes (Link) Fries indefinitely without ever forming plasmodium. Kerr (1960) hypothesised that brucine acted as a chelating agent which removed the multivalent cations necessary for fusion of the myxamoebae. The work of Abe (1933) and of Kerr and Sussman (1958) support the first theory. Abe postulated a difference in electrostatic charge, and Kerr and Sussman the presence of multivalent cations representing a collective a molecular conformation. The second theory is more amenable to testing as it postulates a limited number of biochemically and serologically analyzable entities. Gregg (1961), in an immunoelectrophoretic study of the cellular slime mold Dictyostelium discoideum, detected different antigenic determinants as the organism completed its life cycle. The maximum number of detectable surface antigens possessed by the vegetative amoebas, migrating pseudoplasmodia, and mature spores were determined to be nine, ten and twelve, respectively. When starved, individual vegetative amoebae aggregate in response to a to a chemotactic signal. The aggregating amoebae form long chains which flow together forming a grex, or pseudoplasmodium, whose integrity depends upon strain-specific cell adhesion and coordinated morphogenetic movement (Gerisch, 1976). The chemotactic signal for D. discoideum was identified as adenosine -3', 5' monophosphate (cyclic AMP) (Konijn et al., 1967). Low concentrations of cyclic AMP

cause amoebae to aggregate. This substance acts on the amoebal cell surface, polarizing the direction of pseudoplasmodial activity, directing it toward the highest concentration of cyclic AMP. D. discoideum is one of only a few species found that produce substantial amounts of cyclic AMP and employ it as an aggregating signal in development (Bonner, 1967). The cyclic AMP may possibly by the mechanism that exposes, expresses or interacts with the different detectable surface antigens. This would support the second theory of cell-cell contact exchange. The cellular slime molds are not the only organisms which use a specific signal in development. In certain yeasts and other fungi, individual cells do not sexually differentiate (become gametes) until induced to do so by a sex hormone or specific chemical (for a review see Carlile, 1976). However, once differentiated, their mating is quite particular, i.e., under normal conditions a cell will fuse only with a cell of the opposite mating type, and that cell must be of the same species. Homothallic and interspecific crosses are difficult to obtain. This indicates the presence of mating type and species-specific substances on the surface of the yeast cells which identify their sexual species. In the heterothallic yeast, Hansenula wingei (Crandall and Brock, 1969), and in the green algae, Chlamydomonas eugametos (Wiese, 1973), opposite mating types exhibit sexual agglutination when brought together in culture.

Brock's explanation of the agglutination is that a specific substance (probably a protein) is present on the surface of one of the mating types and combines with a specific substance (probably a polysaccharide) present on the surface of the opposite mating type. The fungal and algal sex attractants that have been partially or completely characterized are secondary metabolites produced after the exponential growth phase. Secondary metabolites are often associated with differentiation, but few have any apparent function (for a review see Carlile, 1976). Similar mechanisms may be involved in the specificity of parasitic and symbiotic associations between plants and animals (Callow, 1975); binding of bacteriophages to bacterial cell walls (Lindberg, 1973); conjugation of bacteria (Sermonti, 1969); acceptance or rejection of the pollen grain on the stigma surface (Linskens, 1969; Knox et al., 1972) and of the binding of sperm cells on the egg cell of animals (Metz, 1969); the specific aggregation of sponges (Henkart et al., 1973) and the specific cell recognition and attraction of compatible myxamoebae fusing to form a zygote in the Myxomycetes.

One approach to studying the specific cell surface differences associated with myxamoebal fusion is to use immunological techniques. Franke (1967) used double diffusion and immunoelectrophoretic analysis of plasmodial extracts to examine the taxonomic relationships of

myxomycetes in the order Physarales and found that serological relationships do exist among the species. In most cases the relationships among the species tested do not coincide with current taxonomy based on morphology of fruiting bodies. The isolates showed strong serological affinity but were not consistent from strain to strain. Kuhn (1890) working with P. polycephalum investigated the nature of the cell surfaces on amoebae and plasmodia using immunofluorescence and double diffusion tests. No strain-specific antigen at either the amoebal or plasmodial stage was found. However, common and stage-specific antigens were observed in the double diffusion tests.

The purpose of my study is two-fold. The first is to examine the compatible mating strains of the Myxomycetes using fluorescent antibody techniques to determine if they possess species-specific antigenic determinant sites. A direct fluorescent antibody (FA) technique was chosen because (1) it is useful in fluorescent inhibition studies and (2) because it is valuable as a diagnostic tool. FA is a specialized immunological technique which consists of an antigen-antibody reaction made visible by the incorporation of a fluorescent dye. In the direct method, the fluorescein isothiocyanate (FITC) is bound directly to the antibody which binds the antigen and forms a fluorescent complex. This linkage when done properly does not alter the immunological reactivity or specificity of the antibody (Coons et al.,

1941). Demonstrating the presence of species-specific antigenic determinant sites may aid in the understanding of the mechanisms involved in cell contact interactions.

The second phase of my study was designed to examine the mating type and species relationships through electrophoresis of protein extracts from myxamoebae. A disc electrophoresis on sodium dodecyl sulfate (SDS) - polyacrylamide gel was chosen because of its extensive use in the identification and characterization by electrophoretic patterns of closely related species (Vaughan and Denford, 1968; Johnson, 1969; Ladizinsky and Adler, 1975; Ladizinsky, 1979).

Protein electrophoresis has become a useful tool in evolutionary studies as an additional approach to assess species relationships (Shechter, 1975; Waines, 1975). For example, electrophoretic separation of proteins has been used to show significant or distinct differences between strains of Myxomplasma hominis at the subspecies level (Razin, 1968; Hollingdale and Lemcke, 1970).

Electrophoresis is the migration of ionic material in an electrical field. Disc electrophoresis employs a discontinuous pH system which has the capacity for concentrating the ions into a narrow band. Polyacrylamide is commonly used as the support medium for disc electrophoresis because the degree of crosslinking can be controlled, thereby improving the separation of the ions. Sodium dodecyl sulfate (SDS) is an anionic detergent that denatures the

protein by irreversibly binding to the protein and stretching it out. Separation is dependent on the molecular weight of their polypeptide chains (Weber and Osborn, 1969; Collins and Haller, 1973). The polyacrylamide gel is formed in a quartz tube (150 x 7mm, 5mm ID) and is composed of three layers. The first layer, the sample buffer mixture (pH 7.2) holds the sample. The second layer, the stacking gel (pH 6.8), concentrates the sample. The third layer, the separating gel (pH 8.6) sieves and separates the sample.

Two species of Myxomycetes, Physarum polycephalum and Didymium iridis, and one species of Acrasiales (or cellular slime mold) Dictyostelium discoideum were used in the study. P. polycephalum was used because nearly all the physiological studies using Myxomycetes have been based on this species (see Hawker, 1952; Alexopoulos, 1963; Gray and Alexopoulos, 1968 for a review). It was one of the first species to have its life cycle worked out in detail (Wilson and Cadman, 1928; Howard, 1931; Gilbert, 1935; Gutes, Gutes, and Rusch, 1961). It is easy to obtain and cultivate, and it can be cultured axenically on a defined medium (Daniel and Baldwin, 1964). D. iridis was used because its mating system has been studied genetically (Collins, 1961; Alexopoulos, 1963). The heterothallic slime mold D. iridis follows the classical sexual pattern of syngamy between pairs of cells (Ross, 1967) or alternatively follows an inducible pattern of multiple fusion of cells and nuclei to

yield syncytia resembling those found in neoplasias and virus induced heterokaryons (Gray and Alexopoulos, 1968). Dictyostelium discoideum was chosen as the single representative of the acrasiales because of the vast amount of literature on the species. It has been studied immunoelectrophoretically and is easy to obtain and culture.

PHASE I

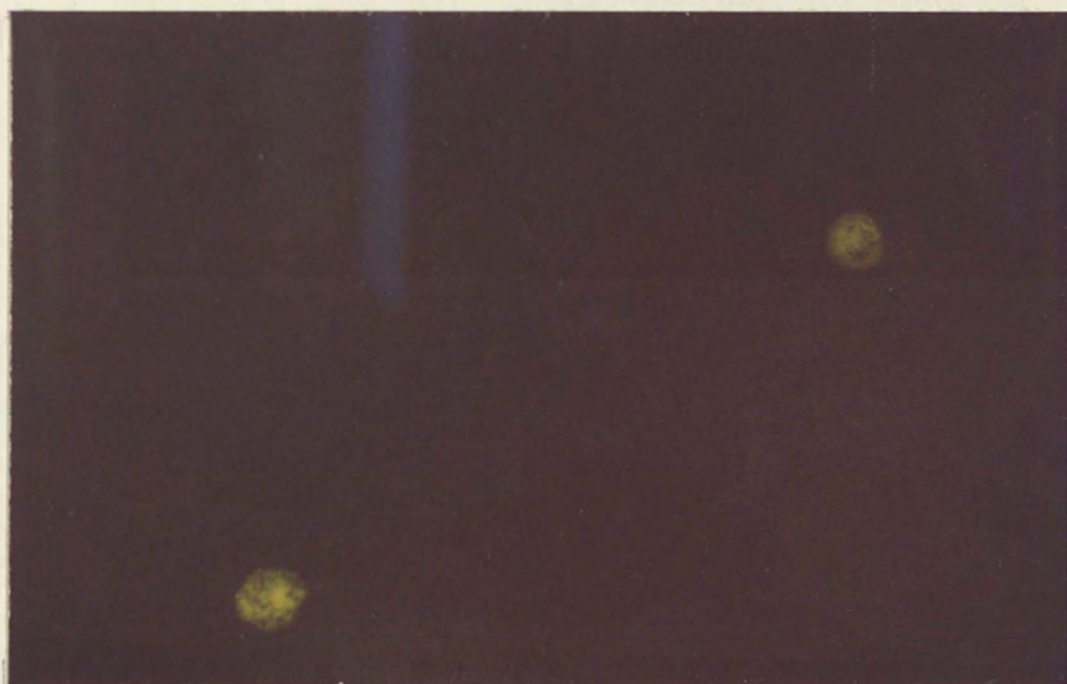
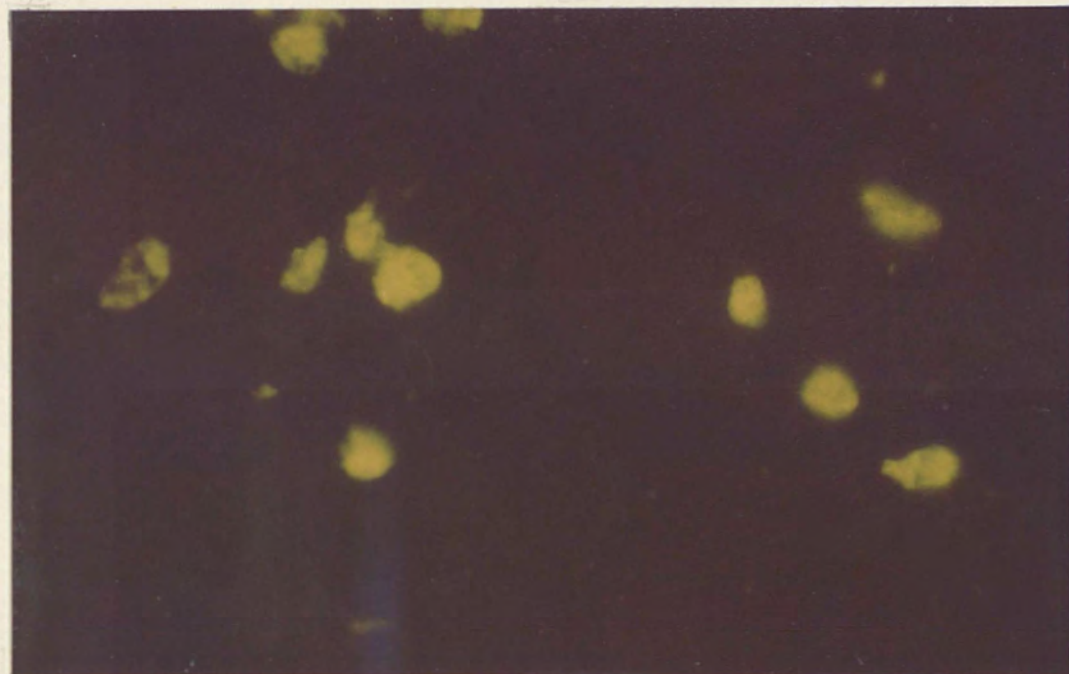
Production of Specific Fluorescein Isothiocyanate-Labeled-Globulins to Physarum polycephalum and Didymium iridis Myxamoebae

Materials and Methods

Strains

The P. polycephalum myxamoebal clones used were obtained from Roger Anderson, Massachusetts Institute of Technology, Cambridge. Two clones were used, LU 647 (mat A1 mat B1 fus A2) and LU 688 (mat A2 mat B1 fus A1). In describing the loci on P. polycephalum: mat A is a tentative suggestion for the redesignation of mt, mat B is the designation for a new locus which affects mating, and fus A is a locus affecting plasmodial fusion. LU is a heterothallic (mt₁) strain derived by a backcrossing to CL a mt₁ progeny from the cross. The D. iridis isolates were from two sources, Honduran 1-2 (mtA1) and Honduran 1-7 (mtA2) were obtained from O.R. Collins, University of California, Berkeley and the other strain HP-147 (mtA2) was obtained from Greg Shipley, University of Florida, Gainesville. The Dictyostelium discoideum, a cellular slime mold was obtained from H.C. Aldrich, University of Florida, Gainesville. See Figure 3 for an example of a typical immunofluorescence.

Fig. 3. Example of immunofluorescence.
Top - Didymium iridis Hon 1-2 (400x)
Bottom - Physarum polycephalum LU647 (600x)



Cell growth

Myxamoebae were grown on solid DS/2 media (D-glucose, 1.0g; yeast extract, 0.5g; MgSO₄, 0.2g; K₂PO₄, 1.5g; and agar 15g/liter deionized H₂O) in a two membered cultured with Escherichia coli ATCC #25922 as the nutritive bacteria. The E. coli was grown in liquid DS/2 at 30°C. The E. coli cells were transferred to the solid DS/2 plate with a pipette. The myxamoebae were transferred using a transfer loop. The mixed suspension of E. coli cells and the myxamoebae were incubated at 23°C in the dark for 42 hr prior to harvesting.

Harvesting

Harvesting of the myxamoebae was done by pouring 5ml of cold (5°C) Bonner's salt solution (NaCl, 0.6g; CaCl, 0.3g; deionized H₂O, 1000ml) onto the agar, gently dislodging the myxamoebae from the agar surface with a sterile bent glass rod, and pouring the myxamoebal suspension into a conical centrifuge tube. Approximately 10ml of the chilled suspension of myxamoebae were centrifuged in an International Refrigerated Centrifuge model B-20 with a SPX head at 100 x g for 5 min. The supernatant was poured off and the precipitated cells were then resuspended in cold Bonner's salt solution and centrifuged again. This process was repeated three to five times with decreasing volumes to yield a cell mass of approximately $8 \times 10^5 - 2 \times 10^6$ cells/ml

(Konijn and Raper, 1961). (See Fig. 4 for a flow diagram of the procedure.)

Growth curve

The number of myxamoebae were determined from day to day using a hemocytometer and a Coulter counter model ZB. Six 0.1mm^2 hemocytometer grid squares were counted/sample and averaged to yield the number of myxamoebae/ml. Sizing of the myxamoebae was required to set the threshold on the Coulter Counter. The diameters of 100 myxamoebae/strain were measured using an eyepiece micrometer. The myxamoebae were found to have a size range approximately the same as a human red cell (6mm to 13mm) enabling the use of a control serum (4°C) to check the efficiency of the Coulter Counter. Preliminary tests with Bonner's salt solution (Bonner, 1947) as a diluent on the test myxamoebae had high background counts which were unacceptable (over 100 units). Isoton II was substituted for the Bonner's salt solution in harvesting and dilution in order to get background counts down to an acceptable range. Dilution of the myxamoebae were accomplished by Diluter II in order to get the counts down to a statistical range. Growth curves for all strains were determined (Figs. 5, 6) so that all cultures of growing myxamoebae were harvested in the exponential growth phase ($8 \times 10^5 - 2 \times 10^6$ cells/plate). This method ensured amoebal population that were essentially free of encysted

Fig. 4. Flow diagram for production of specifically labeled globulins.

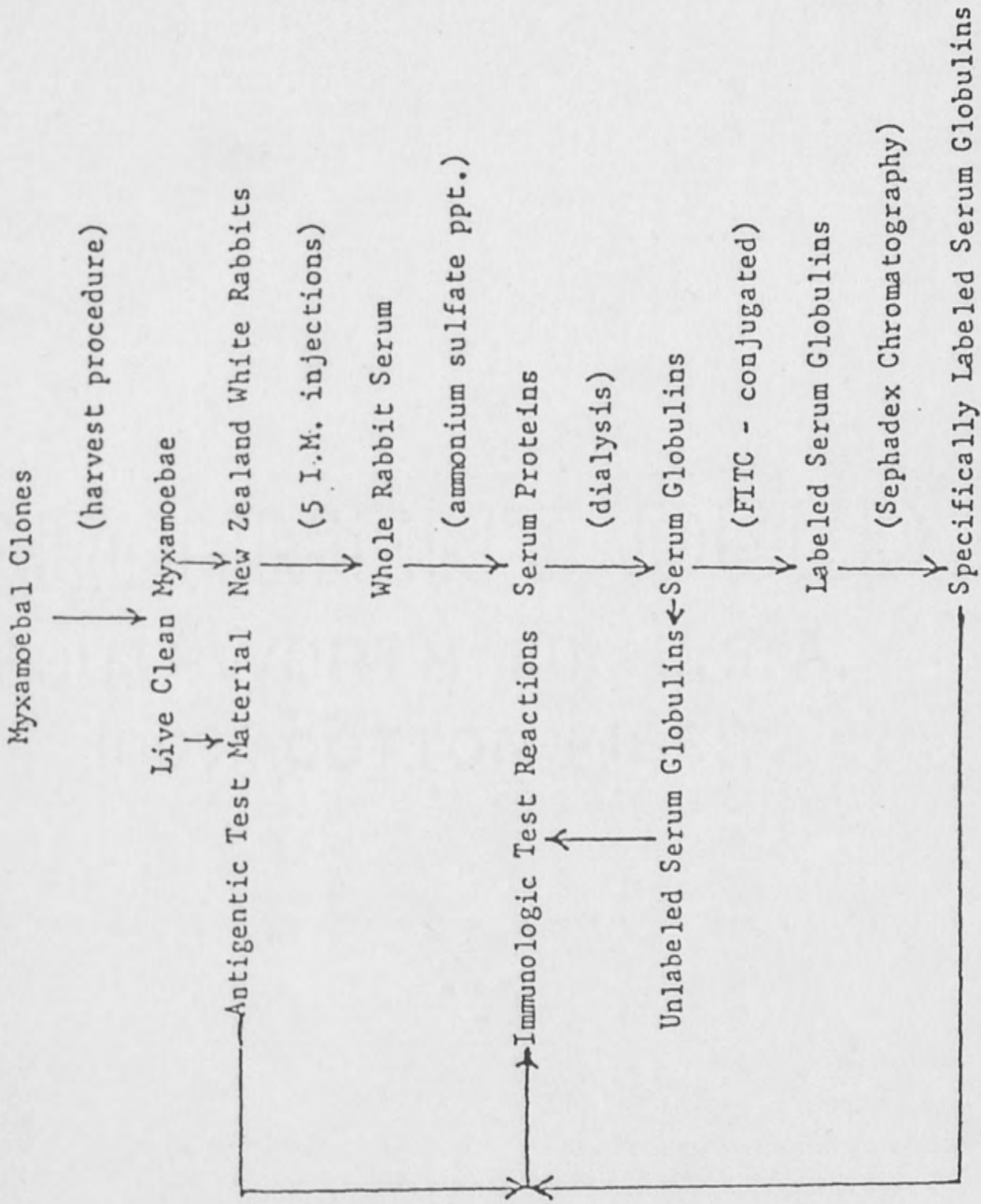


Fig. 5. Growth curve for the Myxomycetes, □ D. iridis Hon 1-2, ■ D. iridis Hon 1-7, ○ D. iridis HP-147, ▲ P. polycephalum LU647, ▲ P. polycephalum LU688, as determined by using a hemocytometer.

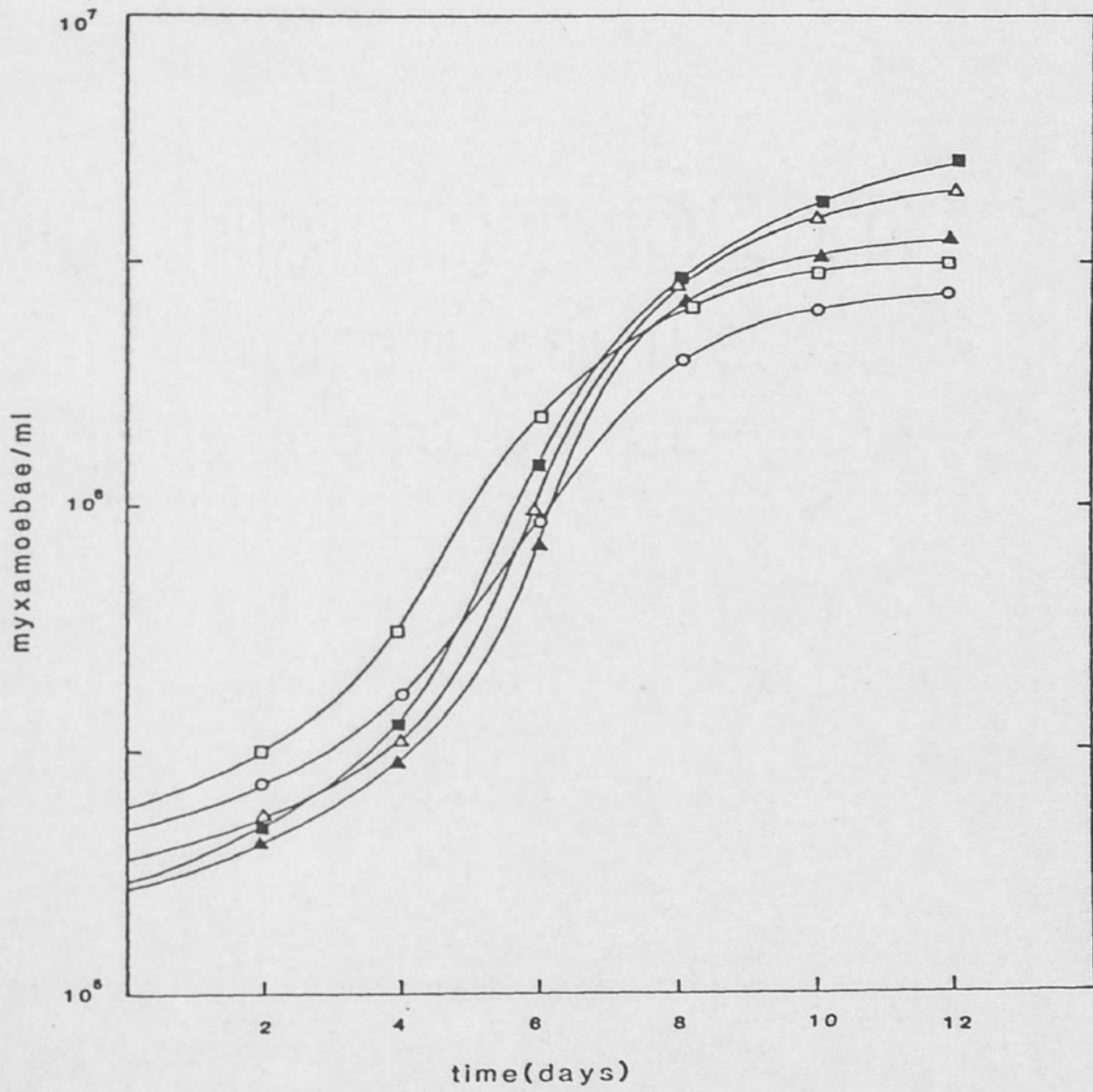
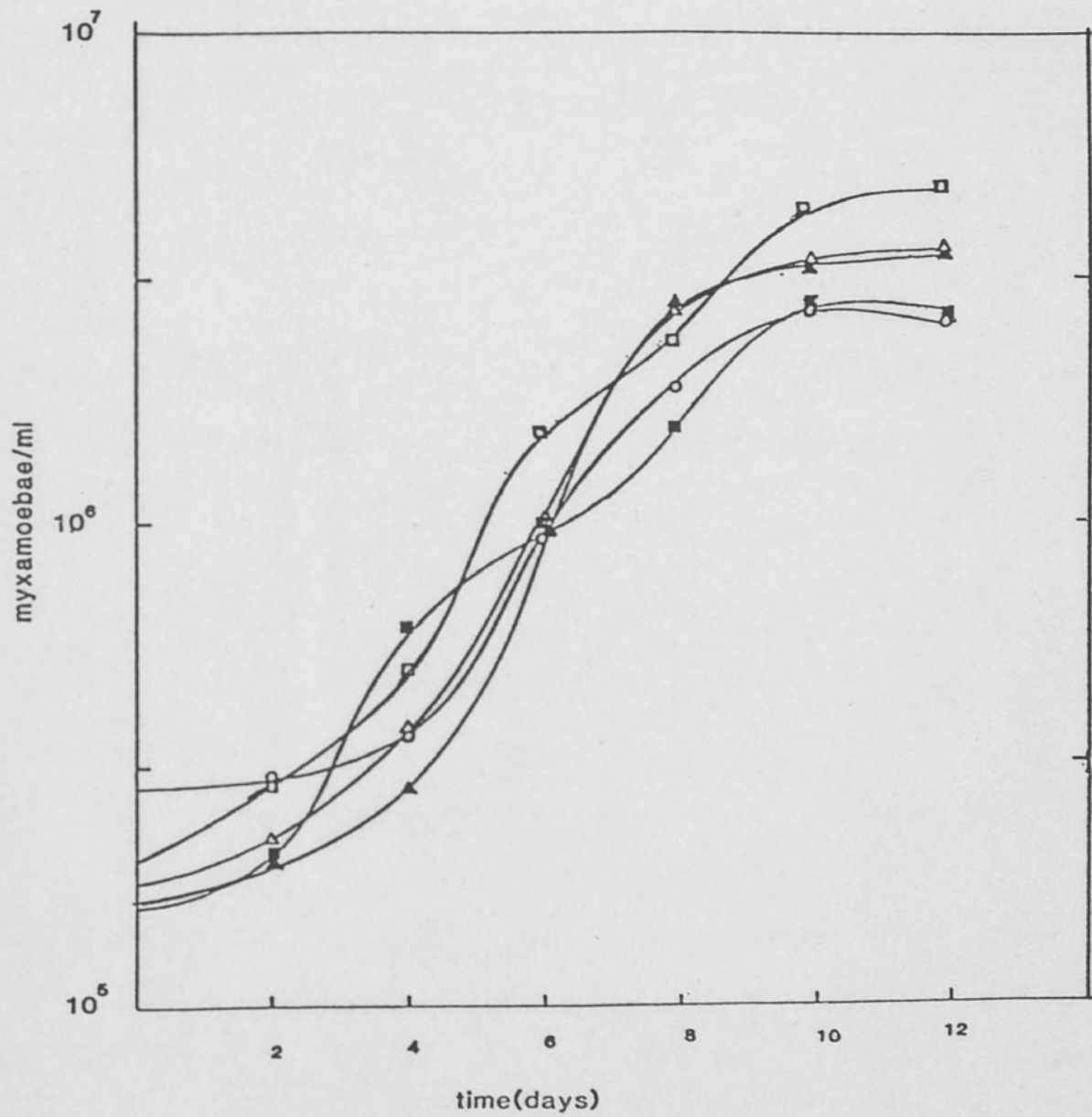


Fig. 6. Growth curve for the Myxomycetes, □ D. iridis Hon 1-2, ■ D. iridis Hon 1-7, ○ D. iridis HP-147, ▲ P. polycephalum LU647, ▲ P. polycephalum LU688, as determined by using a Coulter Counter.



amoebae since encystment starts in the leveling off phase (Kuhn, 1980). Mating and transition to the plasmodial form are threshold phenomena occurring at amoebal densities above 5×10^5 cells/plate. Therefore, it was thought that if amoebae in clonal culture express mating type antigens at a restricted time, it would be at these densities.

Preparation of anti-myxamoebal antibodies

New Zealand white rabbits were immunized with each strain of P. polycephalum and D. iridis. Two rabbits were used for each immunizing strain. One rabbit was injected with a myxamoebal suspension ($1-2 \times 10^6$ myxamoebae/ml in Bonner's salt solution directly from the harvest procedure, and the other was injected with a myxamoebal suspension in Freund's incomplete adjuvant. Two ml myxamoebae were emulsified in 2ml of incomplete Freund's adjuvant. The antigenic material was prepared fresh each time prior to injection. The antigens were injected intramuscularly (IM), 0.5ml to each hind leg, five injections with 5 day intervals between injection and boosted every 30 days for 90 days after the final 5 day interval. Blood was harvested and serum removed 7 days after the fifth injection and 7 days after the final 30 day booster by ear laceration, aspiration and centrifugation. This was stored at -10°C .

Titration with the microtiter apparatus

The microtitration apparatus consists of a lucite

plate, a micropipette, and a microdiluter (Cooke Engineering Co.). The system used is made up of the antigen (myxamoebae, $8 \times 10^5 - 2 \times 10^6$ cells/ml, harvest procedure described above) the antisera, (heated at 56°C for 30 min to destroy the complement in the rabbit serum), the diluent (.01m phosphate buffered salt solution (PBS)), and 2% (v/v) human RBC suspension (M. Sweeney, personal communication). The RBC suspension was shown to be a mechanical trapping, instead of an agglutination or neutralization, by the button formed in the control (antisera and 2% (v/v) RBC suspension). A micropipette was used to dispense 1 drop (0.025ml of diluent into all the wells in the lucite plate. One row (12 wells) were allowed for each sample, one row for positive control, and one row for negative control. Antiserum (0.025ml) was then added to first well and diluted across the row serially with a 0.025ml microdiluter (1:2 to 1:2048). Antigen (0.025ml) was added to every well of the rows. A 2% (v/v) human RBC suspension was added to every well to serve as an indicator. The plates were covered with a transparent tape to prevent drying, mixed, and incubated at 37°C for 1 hr and at 23°C overnight. The endpoint of the titration was determined by the pattern formed by the cells as described by Landsteiner et al. (1941). The tray was first observed with the Microtiter mirror stand and then quantified using a compound light microscope. The individual wells were compared to the

negative control (antigen diluent, and RBC's) and rated (0 to +4).

Isolation of globulin fraction from rabbit anti-myxomycetes whole serum

Fractionation and purification of the five different pooled sera was accomplished using Engval's and Perlmann's (1971) method. An amount of 70% (w/v) ammonium sulfate at pH 7.2 equal to the serum was added dropwise and with constant stirring at room temperature to the immune serum so that the final concentration of $(\text{NH}_4)_2\text{SO}_4$ was 35%. The mixture was allowed to stir for 30 min at 25°C and then was centrifuged at 1000 x g for 30 min at 20°C. The supernatant fluid was discarded and the precipitate was washed in one volume of 35% $(\text{NH}_4)_2\text{SO}_4$ and centrifuged as above. The precipitate was resuspended in a small volume of sterile deionized water (2ml) and placed in dialyzing membranes. The globulins were dialyzed against pH 8.0, 0.85% NaCl at 5°C with slow stirring. The saline dialysate was changed 3 times in a 48 hr period. The presence of $\text{SO}_4^{=}$ was checked by the addition of saturated barium hydroxide.

Preparation of fluorescein-isothiocyanate labeled antibodies

The concentration of proteins and the amount of FITC necessary for conjugation was determined by measuring the O.D. of the serum at 280nm and 495nm and using the following formula: proteins (mg/ml) = $0.35 \text{ O.D. } 280\text{nm} - 0.35 \text{ O.D. } 495\text{nm} \cdot 1.4^{-1}$

according to the method of Wood et al. (1965). The globulin fraction was added to equivalent amounts of PBS (.1M pH 9.5) and FITC (10mg of FITC/g of protein). Conjugation was allowed to proceed with rapid stirring, at 4°C for 21 hours. The conjugated mixture was separated from excess FITC by column chromatography (Sephadex G-25). Fractions were collected using 0.13M PBS at a flow rate of 1.5 ml/min. Elution of the protein was monitored by measurement of absorption at 280nm on a Beckman double beam spectrophotometer. The specific FITC labeled globulins were divided up into 1 ml fraction and stored at -10°C for latter use.

Preparation of antigenic test material

Harvested myxamoebae for immunological studies were suspended in distilled water and used as antigenic test material. Precleaned (70% ethanol) slides were used for preparing heat fixed and wet-mount antigen slides. One or two drops of antisera were applied to the fixed preparations and these were incubated in a moist chamber for 30 min at 37°C. After incubation the slides were washed twice for 5 min each in PBS, dipped in deionized water, and air dried. Preliminary titration studies showed that the intensity of fluorescence was greater (fluorescence of myxamoebae to background) when the FA was diluted 1:20 to 1:40. Wet mounts were prepared from antigen samples which were incubated in the presence of labeled antibody in centrifuged

tubes for 30 min at 37°C and subsequently washed by centrifugation (1,000 x g) in distilled water. Number 1 cover glasses (24 by 40mm) were mounted with a medium consisting of 90% glycerol and 10% PBS.

Fluorescent - inhibition (FIH)

Slides for fluorescent inhibition (FIH) test were fixed as outlined in the preceding paragraphs and exposed to unlabeled antibody for 30 min at 37°C in a moist chamber. The FIH slides were washed 2 times in 0.13M PBS and distilled water to remove unbound antiserum. They were reincubated for 30 min at 37°C in a moist chamber in the presence of labeled antibody and then washed, as previously described. The specificity of the labeled antibody was demonstrated by the ability of the unlabeled antibodies to block the fluorescent activity. Fluorescent antibody testing in my study was the first to demonstrate the presence of myxamoebal antigens.

Antisera adsorption

Cross reactivity of each labeled antisera was tested by heteroabsorption. Heteroabsorption consists of removing all the common specificities from an antiserum by adsorption with heterologous amoebae, leaving in the antiserum only specificities unique to the homologous cell. The antisera was incubated with the heterologous amoebae for 30 min at 37°C and subsequently washed by centrifugation (1000 x g) in

distilled water. After adsorption by heterologous amoebae, the activity of the serum was tested by immunofluorescence against homologous amoebae. If the strain expressed a unique kind of antigen in sufficient quantity, the antigen, would be detected by immunofluorescent heteroabsorption. Fluorescent microscopy was performed on a Zeiss model photomicroscope using a Zeiss power supply and 100-W light source.

PHASE II

Identification and Characterization of Physarum polycephalum, Didymium iridis, and Dictyostelium discoideum by Disc Electrophoresis on Sodium Dodecyl Sulfate-Polyacrylamide Gel

Material and Methods

Strains

The three species, Physarum polycephalum, Didymium iridis, and Dictyostelium discoideum used in this study are identical to those used in Phase I of this study. Growth, nutrition, and harvesting of the myxamoebae was as described previously (Konijn and Raper, 1961).

Preparation of test material

The washed myxamoebal pellet was then suspended in Bonner's salt solution to yield a population of $2-8 \times 10^6$ cell/ml (Fig. 6) and sonicated using a Sonicator Cell Disruptor model #200R. Sonication was carried out in 1 min intervals (Microtip's set at 6, 40% duty with continuous output) in an ice bath until the myxamoebae were completely disrupted as determined by microscopic observation. The myxamoebal protein was then concentrated using trichloroacetic acid (TCA) precipitation. The TCA precipitation was carried out in a cold room (4°C) using a 50% TCA solution.

The 50% TCA was added to the myxamoebal protein to yield a final TCA concentration of 10%. The preparation was allowed to stand over night at 4°C for the precipitate to form. The precipitate was centrifuged at 1,000 x g for 30 min. The supernate was aspirated off, and the precipitate was washed two or three times with cold acetone, air dried, and resuspended in 0.01M PBS, pH 7.2. The protein concentration was determined using the method described in I. The protein preparation was diluted with 0.1M PBS, pH 7.2 to 1mg/ml and stored at -15°C.

Preparation of the gel

A 10% polyacrylamide - SDS gel was made (Blatter et al., 1972). Recipes for the gels, buffers, stains, and destaining solution are given in Table 1. The separating gel was mixed and (150 x 5mm ID) quartz tubes were filled to the 3/4 mark. The tubes were then overlaid with water and allowed to polymerize for 1 hr. The stacking gel was then prepared, the water overlay removed from the separating gel and the stacking gel was then placed into the tubes to a predetermined mark. The tubes were again overlaid with water to avoid a meniscus formation of the gel and allowed to polymerize 1 hr. After polymerization the tubes were placed in a gel electrophoresis cell (Bio-Rad model #150) with a electrophoresis constant rate power source (Canalco model # 300B). The protein sample was then mixed with the

TABLE 1

Recipe for 10% Polyacrylamide-Sodium Dodecyl Sulfate Gel

Running Gel

10.0	30	%	Acrylamide
3.9	1	%	Methylenebisacrylamide (Bis)
8.1	1.5M		Tris (hydroxy-methyl) aminomethane pH 8.7
.15	20	%	SDS
7.9			H ₂ O
.01			N,N,N',N'-tetramethylethylenediamine (TEMED)
.1			Ammonium persulfate (10%)

Stacking Gel

1.67	30	%	Acrylamide
2.6	1	%	Bis
1.25	0.5M		Tris pH 6.8
0.05	20	%	SDS
4.4			H ₂ O
0.005			TEMED
0.05			Ammonium persulfate (10%)

Sample Buffer Mixture

0.0625	0.4M		Phosphate buffered saline pH 7.2
0.25	20	%	SDS
0.125			β -mercaptoethanol (100%)
0.125			Glycerol-bromo phenol blue (9:1)

Running Buffer pH 8.6

6.0g			Tris
28.8g			Glycine
5.0g			SDS
			H ₂ O to 1000 ml

(dilute 1:4 for use.)

Reaction Mixture

30	1%		sample buffer mixture
70	1%		protein in 10 ⁻³ M PBS pH 7.2

(Bring to boil for 2 minutes)

Stain

1.25g			Coomassie Brilliant Blue. (R-250)
454	ml		Methanol (50%)
46	ml		Glacial Acetic Acid

Destain

75ml			Glacial acetic acid
50ml			Methanol
875ml			H ₂ O

(Dilute 1:1 with H₂O before use.)

Note: Adapted from Blatter et al., 1972
All measurements are in ml unless otherwise noted.

sample buffer mixture and brought to a boil for 2 min. This facilitated the mixing of the protein with the sample buffer mix. This mixture (0.1ml) was then placed in the tubes. The samples were then run at 2.5 mA/gel for 20 min, then 5mA/gel for 4.5 hr. A tris (hydroxy-methyl) aminomethane-glycine (pH 8.6) running buffer was used.

Gel scanning

The samples were removed from the gel electrophoresis cell and were scanned using an ISCO gel scanner (Instrument Specialties Company model #659). This system allowed the gel to be scanned for absorbance in the ultraviolet (UV) region of the spectrum while still in the tube, without excessive background absorbance from either the tube or the gel. The round gel tube was used as a cuvette with the absorbance measured at 280nm. There are several advantages in the use of the gel scanner. First, there is no danger of contamination by dust, since the transfer operation is eliminated. Second, the gel remains in the running tube during scanning and may be run further after the scan is made (Morris and Wright, 1975).

Removal of gel and staining

After the scan was made, the gels were removed from the quartz tubes with the aid of a pipette bulb and teasing needle. The gels were placed in a #18 test tube (Purex) and stained with coomassie brilliant blue (R-250) for 1 hr. The

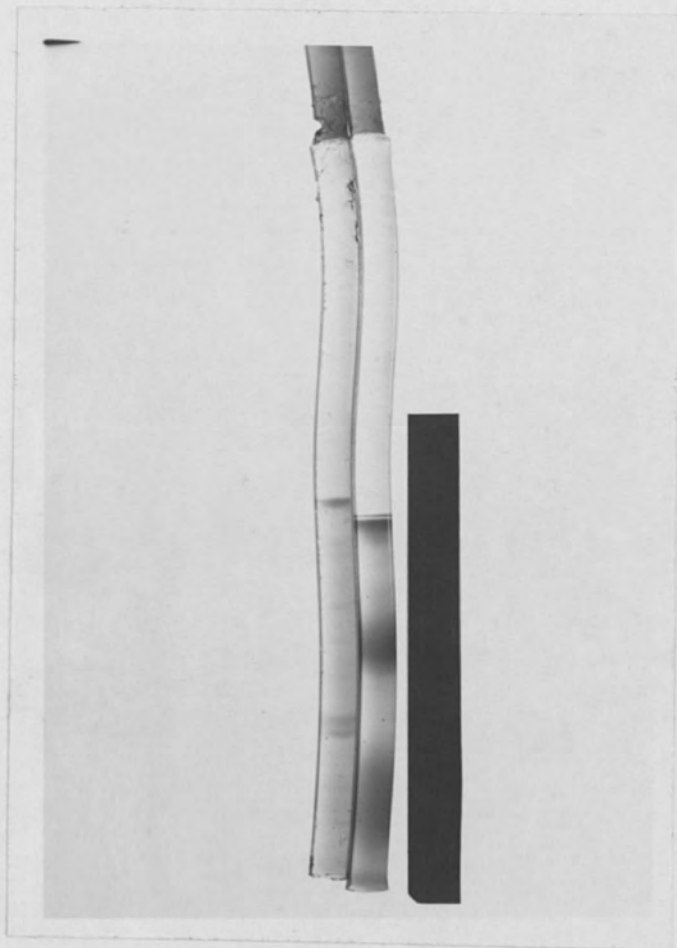
stain was then aspirated off and the destaining solution placed in the tube. This solution was changed periodically (3 volumes/tube) until the bands appeared (approximately 8 days) (see Fig. 7).

Molecular weight determination

Molecular weight determinations were approximated by referencing to a standard. Daltion Mark V (Sigma Chemical Company), the standard used in this procedure, is a mixture of lysozyme (14,300), B-lactoglobulin (18,400) trypsinogen PMSF treated (24,000), pepsin (34,700), egg albumin (45,000), and bovine albumin (66,000).

Fig. 7. Example of electrophoretic patterns
of Myxomycetes.





Tube on left is Didymium iridis HP 147 A², tube on right is Dalton Mark V (standard), and block is 3 inches in length.

RESULTS

Phase I

Amoebal antigens

Similar reactivity was found with each anti-myxamoebal serum (fluorescent antibody) tested against both homologous and heterologous myxamoebal strains. The fluorescence was quantified from 0 to +4, with 0 being negative and +4 giving the greatest fluorescence (see Table 1). The myxamoebal strains to which antisera was produced are mating type alleles, i.e., the strains differ at the mating type locus. These differences were expected to stand out most sharply in comparisons of the antisera. However, amoebal antigenicity appeared to be independent of mating type genotype.

Heteroadsorption of each anti-myxamoebal serum followed by testing serum activity using immunofluorescence showed no reactivity remained. This method suggested that there is no detectable strain-specific activity in any of the anti-myxamoebal sera. The sera was shown, however, to be genus specific (see Table 2). Photomicrographs in Figure 3 were taken by Dr. Carl Fleurman, E.I. Dupont de Nemours, Savannah River Plant and Dr. James L. Koevenig, University of Central Florida.

TABLE 2

Staining and Heteroadsorption of FITC-labeled Sera

FAB	CELLS*					
	A	B	B-1	X	Y	D. discoideum
<u>DIRECT STAINING</u>						
FAB A	+4	+4	+4	+4	+4	+4
FAB B	+4	+4	+4	+4	+4	+4
FAB B-1	+4	+4	+4	+4	+4	+4
FAB X	+4	+4	+4	+4	+4	+4
FAB Y	+4	+4	+4	+4	+4	+4
<u>ABSORPTIONS</u>						
FAB A+A	0	0	0	0	0	0
FAB A+B	0	0	0	0	0	0
FAB A+B-1	0	0	0	0	0	0
FAB A+X	+4	+4	+4	0	0	0
FAB A+Y	+4	+4	+4	0	0	0
FAB B+A	0	0	0	0	0	0
FAB B+B	0	0	0	0	0	0
FAB B+B-1	0	0	0	0	0	0
FAB B+X	+4	+4	+4	0	0	0
FAB B+Y	+4	+4	+4	0	0	0
FAB B-1+A	0	0	0	0	0	0
FAB B-1+B	0	0	0	0	0	0
FAB B-1+B-1	0	0	0	0	0	0
FAB B-1+X	+4	+4	+4	0	0	0
FAB B-1+Y	+4	+4	+4	0	0	0
FAB X+A	0	0	0	+4	+4	0
FAB X+B	0	0	0	+4	+4	0
FAB X+B-1	0	0	0	+4	+4	0
FAB X+X	0	0	0	0	0	0
FAB X+Y	0	0	0	0	0	0
FAB Y+A	0	0	0	+4	+4	0
FAB Y+B	0	0	0	+4	+4	0
FAB Y+B-1	0	0	0	+4	+4	0
FAB Y+X	0	0	0	0	0	0
FAB Y+Y	0	0	0	0	0	0

* A D. iridis Hon 1-2B D. iridis Hon 1-7B-1 D. iridis HP-147X P. polycephalum LU 647Y P. polycephalum LU 688

Antisera titer

The antisera was titered using the microtiter apparatus. It was found to average 1:256 agglutinating units initially and increased appreciably in the final bleed to 1:1024 agglutinating units. The additions of the 2% (v/v) RBC addition to the test allowed the titer to be determined without the use of a microscope. The results (Table 3) show that the use of Freund's incomplete adjuvant did not increase the titer significantly over the IM injections in saline.

Growth curve

Growth curves for the five strains (Figs. 5 and 6) show a typical sigmoidal growth pattern. Although plots of both methods, the Coulter Counter and hemocytometer, yielded a smoother, typical curve. The exponential growth phase was shown to give the desired cell population between the fifth and seventh day (Fig. 5). Comparison of the Coulter Counter and hemocytometer results, on a daily basis, showed that the data varied approximately 5 - 7%.

Phase II

Electrophoretic patterns

Each species examined possessed a characteristic pattern (Fig. 7). Intergeneric differences and similarities in the electrophoretic patterns were also noted when P. polycephalum, D. iridis, and D. discoideum were compared

TABLE 3

Titer Determination of Rabbit Anti-Myxomycetes Sera

	Antibody titer initially ^a	1:2 ⁿ dilution showing agglutination ^b	Antibody titer after booster ^c ,	1:2 ⁿ dilution showing agglutination
<u>D. iridis</u> Hon 1-2	128	7	1024	10
<u>D. iridis</u> Hon 1-7	512	9	1024	10
<u>D. iridis</u> HP-147	512	9	512	9
<u>P. polycephalum</u> LU647	128	7	1024	10
<u>P. polycephalum</u> LU688	256	8	1024	10

^a Initially, indicates the first antiserum harvest (seven days after the final five day interval).

^b Where n represents the number of doubling dilutions (1:2ⁿ) showing agglutination.

^c After booster, indicates the final antiserum harvest (seven days after the final thirty day booster).

(Fig. 8). The number of bands observed in D. iridis Hon 1-2 was shown to be fourteen, while the number observed in P. polycephalum LU647 and D. discoideum was shown to be twelve and nine, respectively. Intraspecific differences were noted in D. iridis. Differences in the electrophoretic patterns produced by these representative isolates of D. iridis are shown in Figure 9. Some of the differences appear to be quantitative rather than qualitative, but at least two of the bands (A and B of Fig. 9) appear to be present in D. iridis Hon 1-2 or Hon 1-7, but not in HP-147. Bands C and D which are present in HP-147 do not appear in Hon 1-2 or 1-7. No other intraspecific differences were noted in D. iridis Hon 1-2 and 1-7 have essentially the same electrophoretic pattern with fourteen bands. This may be due to their extensive backcrossing to CL. In P. polycephalum the two clones have clones have essentially the same electrophoretic pattern with no observable difference (Fig. 10). In D. discoideum nine bands were observed in the electrophoretic pattern of the extracted protein.

Gel scanning

Gel scans on standards (Dalton Mark V) resolved the different protein bands. However, gel scans failed to resolve the different bands on the 10% polyacrylamide - SDS gels containing myxamoebal protein extracts. This is because the extracts electrophoresed in this study contained many

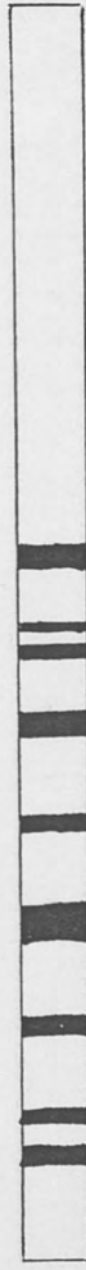
Fig. 8. Interpretive line drawings comparing the electrophoretic patterns of Didymium iridis, Physarum polycephalum, and Didyostelium discoideum.



Hon 1-2 A¹



LU647 mat A¹
mat B¹ fus A²



Dictyostelium
discoideum



Dalton Mark V
(Standard)

Fig. 9. Interpretive line drawings of the electrophoretic patterns of Didymium iridis.



Band A
Band B

Band C
Band D

Hon 1-2 A¹



Hon 1-7 A²



HP 147 A²

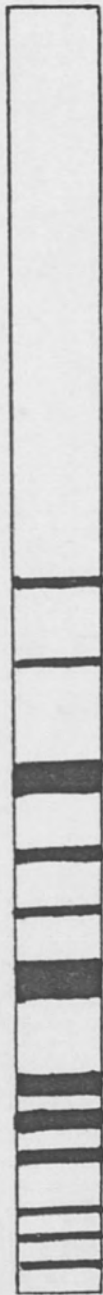


Dalton Mark V
(Standard)

Fig. 10. Interpretive line drawings of the electrophoretic patterns of Physarum polycephalum.



LU647 mat A¹
mat B¹ fus A²



LU688 mat A²
mat B¹ fus A¹



Dalton Mark V
(Standard)

proteins with approximately the same molecular weights. No further scans or changes in gel composition were done after their preliminary findings.

DISCUSSION

The applicability of fluorescent antibody techniques as a rapid reproducible diagnostic tool is well established (Coons et al., 1942; Wood et al., 1965; Nakamura, 1974; Kawamura, 1977). Fluorescent antibody has demonstrated the presence of cell surface antigens on the myxamoebae of P. polycephalum and D. iridis. Kuhn (1980) working with myxamoebae of P. polycephalum found no strain-specific amoebal antigens. My studies confirm the results of Kuhn (1980) in that no strain-specific amoebal antigens were observed in the representative isolates examined. Amoebal antigenicity appears to be independent of genotype. However, the antisera was shown to be genus specific. For example, adsorption of D. iridis antisera with P. polycephalum still gave +4 fluorescence on a scale of 0 to +4 when staining D. iridis myxamoebae, but gave 0 when staining P. polycephalum and D. discoideum. The reverse was also shown to be true when P. polycephalum antisera was adsorbed with D. iridis myxamoebae when staining P. polycephalum, D. iridis and D. discoideum myxamoebae (see Table 2). This finding might be used in the detection and enumeration of Myxomycetes in the soil. More work needs to be done to determine if all of the Myxomycetes are genera-specific. If so, then the genera

of the Myxomycetes could be determined for ecological studies.

In Phase II of this study, disc electrophoresis on SDS-polyacrylamide gel was done as an additional approach to the identification and characterization of P. polycephalum, D. iridis, and D. discoideum antigens. The types of information obtainable from fluorescent antibody and electrophoresis are somewhat different. The pattern of antigens on each cell surface can be visualized and relative antiserum measured by immunofluorescence. If a strain expresses a unique kind of antigen in sufficient quantity, the antigen might be detected by immunofluorescent heteroadsorption. However, if the antigen was covered in some manner, then sonication possibly would uncover the strain-specific antigen, the mechanisms of cell recognition and attraction of compatible mating strains of Myxomycetes is not known. Electrophoretic separation of proteins was chosen because it has been used to show significant or distinct differences between strains at the subspecies level (Raizin, 1968; Hollingdale and Lemcke, 1970).

The finding of intergeneric differences in the electrophoretic patterns of D. iridis, P. polycephalum and D. discoideum supported the finding of genera-specific activities in the antisera. Each species examined possessed a distinct electrophoretic pattern, but only one species, D. iridis, showed significant or distinct differences between strains

at the subspecies (mating strain) level (see Fig. 9). In P. polycephalum the two clones examined have essentially the same electrophoretic mating type. Possible explanations may be as follows:

1. The antigenic differences may be so small that they were undetectable by this method.

2. A compound other than a protein is the antigenic determinant, maybe a polysaccharide.

3. The compound acting as the antigenic determinant requires a stimulus from some other compound or opposite mating type to be expressed (unmasking).

4. The cells possess different glycoproteins, but the techniques utilized in this study remove the sugar side chains and therefore the specificity is lost.

In D. discoideum nine bands were observed in the electrophoretic pattern of the extracted protein. The demonstration of nine bands is consistent with the number of bands that Gregg (1961) observed in vegetative amoebae of D. discoideum in his immunoelectrophoretic studies. The proteins shown in my electrophoretic pattern may possibly be the antigenic determinants observed by Gregg. Further work needs to be done with other Myxomycetes to determine whether the banding patterns are consistent for their isolates. If so, an investigation into the nature and function of the proteins which constitute the atypical bands may give some insight into the determination of the

mechanism of cell-to-cell contact interactions. The possibility exists that these proteins are involved, directly or indirectly in the determination of race specificity. Identification of the proteins could provide a better understanding of the mechanisms of cell-to-cell contact relationships. The techniques used in this study are not new, but the use of fluorescent antibody coupled with protein electrophoresis has proven to be a different approach to the still unanswered question, What causes the cell recognition and attraction of compatible mating strains of the Myxomycetes?

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