A STUDY OF ISOENZYME ACTIVITY IN *DIDYMIUM IRIDIS* DURING SEVERAL DEVELOPMENTAL STAGES OF THE LIFE CYCLE

by

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The author would like to dedicate this thesis to his parents, Michael and Donna Szilagy, whose love, support and understanding made this study a reality.

ABSTRACT

The Myxomycetes are an excellent model for scientific investigations including those that deal with cellular biochemistry and metabolism. Four geographical isolates of Didymium iridis and their respective plasmodia1 phase were analyzed by agarose gel electrophoresis to determine the primary modes of metabolism that occurred developmentally during the major stages of the life cycle.

The glycolytic pathway appears to be quite prominent in the Myxamoeba as supported by the electrophoretic patterns of the enzymes Phosphoglucomutase (PGM) and Glucose Phosphate Isomerase (PGI), with both exhibiting an elevated activity. This pathway would then appear to be of primary importance in this phase of the life cycle not only for energy production but as a means for carbohydrate production through gluconeogenesis. The carbohydrates' moieties would then be made available for encystment which is of frequent occurrence since these cells are extremely sensitive to the depletion of those metabolites essential for growth. Evidence also indicates that the hexose monophosphate shunt is prominent during this stage of the life cycle as demonstrated by the high level of activity of Glucose-6-Phosphate Dehydrogenase (G-6-PDH), which functions to produce important products necessary for DNA synthesis, thus enabling the Myxamoeba as

observed to grow exponentially in preparation for zygote and ultimately plasmodia1 formation. The isoenzyme data seems to support the contention that PGM is a bridging enzyme which permits the interaction of glycolysis and the hexose monophosphate shunt.

The citric acid cycle is the most prominent pathway in the plasmodial stage of the life cycle as demonstrated by the elevated activities of the enzymes Isocitrate Dehydrogenase (ICDH) and Glutamate Oxaloacetate Transaminase (GOT). These enzymes appear to be necessary for an assimilative function of the plasmodia as well as its growth prior to sporogenesis.

The sporulation stage demonstrated, as expected, decreased levels of enzyme activity regarding PGI and Alkaline Phosphatase, as well as the complete absence of other metabolic enzymes. This indicates that this stage represents one of reduced metabolic activity and dormancy.

Finally, electrophoresis of total protein followed by highly sensitive silver staining indicates that there are marked differences regarding structural proteins among the clones used in this study.

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LIST OF ABBREVIATIONS

EDTA (Na_2)	Ethylenediamine Tetra Acetic Acid
MTT	[3-(4,5-Dimethylthiozolyl-2)-2,5 Diphenyltetrazolium Bromide
B-TPN (NADP)	Nicotinamide Adenine Dinucleotide Phosphate
PMS	Phenazine Methosulfate
$MgCl_2$	Magnesium Chloride
NaH_2PO_4	Sodium Phosphate (Monobasic)
Na_2HPO_4	Sodium Phosphate (Dibasic)
Tris	Trizma Base, Reagent Grade
S-0751, C-7254, B-0252, etc.	Examples of Sigma Chemical Company catalog numbers which aid in selecting the correct reagent

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CHAPTER I

Introduction

Electrophoresis continues to be a very important tool in the elucidation and analysis of biological macromolecules. The contribution of electrophoresis to our present understanding of proteins is second to no other method and is well documented in the literature (Goullet, 1980; Casse et al., 1979; and others). Its impact is felt in the areas of biochemistry, physiology and medicine.

The technique of agarose gel electrophoresis has frequently been used in taxonomic characterization of organisms by means of isoenzyme profiles (Cann and Wilcox, 1965). This technique has been used successfully for the identification of various plant pathogens.

Electrophoresis has also been instrumental in defining the taxa of certain fungi. Chang et al. (1962) demonstrated different protein patterns between the wild strain of Neurospora crassa and a mutant strain. Meyer et al. (1964) found that unique esterase and phosphatase patterns were produced from cultured filtrates of formae speciales of Fusarium oxysporum and F. xylarioides. Glynn and Reid (1969) also used protein patterns as a possible means of taxonomically identifying Fusarium oxysporum and

13 other Fusarium species. Fungi successfully identified with disc electrophoresis include three species of Penicillium (Bent, 1967) and seven other species of Aspergillus (Kulik and Broooks, 1970).

Presently, very little appears in the research literature concerning electrophoretic studies of the order Physareles, including Didymium iridis. Some of the earliest research was performed by Zeldin and Ward (1963), where gel electrophoresis was used to detect changes in protein patterns of polysaccharidases in the plasmodia and presporangia stages of Physarum polycephalum. The application of starch gel and polyacrylamide gel electrophoresis has been incorporated in various taxonomic problems concerning the myxomycetes of the order Physareles (Franke et al., 1968, Franke and Berry, 1972). The use of immunoelectrophoresis (Graber and Williams, 1963) and other techniques imply that classification of the myxomycetes may be more efficiently resolved by studying the natural relationships at the molecular level.

Donald A. Betterley and O'Neil Ray Collins from the Department of Botany, University of California, Berkeley, recently published a report on the genetic divergence among the varieties of D. iridis (1983), including those that have the ability to interbreed, as

well as those that do not. Their research indicates that correlation exists between mating type compatibility and isoenzyme polymorphism. These types of studies have also been carried out on various fungi (Spieth, 1975; Baptist and Kurtzman, 1976).

Statement of the Problem

It appears up to this point that most applications of electrophoresis concerning the myxomycetes have been of a taxonomic nature. The intent of this study was to examine the developmental aspects of the myxomycetes, especially those of D. iridis during the life cycle, utilizing the methods of high resolution, agarose electrophoresis and scanning densitometry.

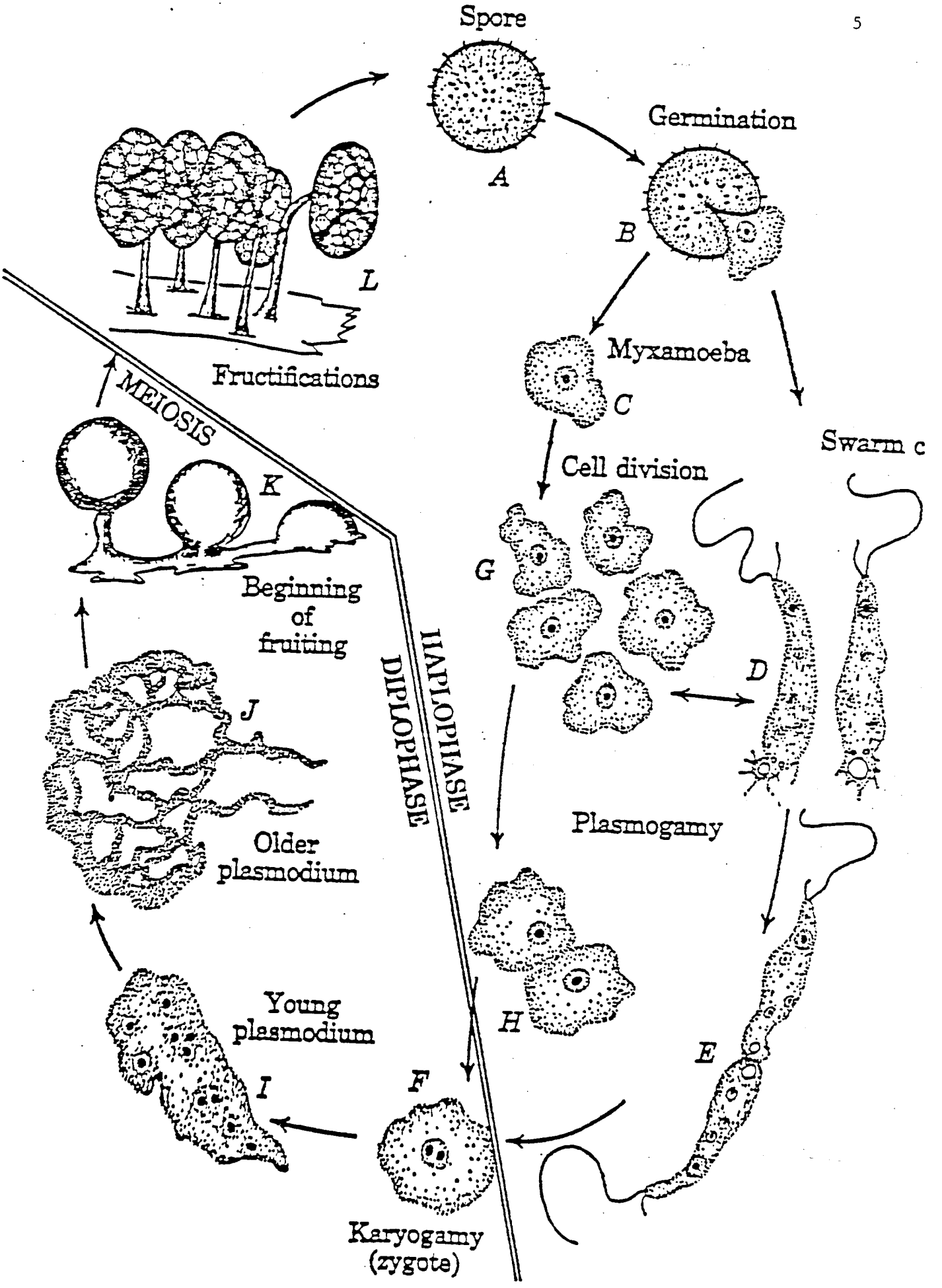
Comparisons were made of the activity and diversity of certain metabolic isoenzymes through the entire life cycle of D. iridis. This will be accomplished by examining the electrophoretic patterns of isoenzymes of the myxamoeba, plasmodium and sporulation stages.

The life cycle of D. iridis (Fig. 1) essentially consists of a myxamoebal haplophase, and a multinucleated plasmodia1 diplophase, and a subsequent sporulation phase.

Myxamoebae arise from the germination of the spore (Collins, 1961). When subjected to a liquid environment, the myxamoeba are transformed into a flagellated swarmer

Figure 1.

Life cycle of a Myxomycete, by C. J. Alexopoulos, 1962, Introductory Mycology, by John Wiley and Sons, Inc., New York p. 72.


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

stage. Unfavorable environmental conditions force the myxamoeba into a protective encystment stage.

Plasmodium formation in D. iridis results from the fusion of two haploid myxamoeba (Dee, 1975), which forms a large syncitium through successive mitotic divisions. Generally a concentration of 10^5 cells must be achieved in order for plasmodium formation to occur (Yemma and Perry, 1985).

Adverse environmental conditions such as dehydration and low temperature (Jump, 1954) transform the plasmodium into a more resistant stage referred to as a sclerotium. The life cycle is completed with the onset of sporulation (meiosis), which normally occurs after starvation (Jump, 1954). Sporulation and sclerotization differ in that the latter is a reversible process that will revert quickly to the plasmodium stage when exposed to a moist temperate environment (Lott and Clark, 1982).

Studies already in the literature concerning the life cycles of D. iridis include senescence of plasmodia (Lot and Clark, 1980; Kerr and Waxlax, 1969), somatic incompatibility (Clark, 1980; Ling and Clark, 1981), and nuclear DNA changes as observed during the life cycle (Therrien and Yemma, 1974; Collins and Betterley, 1982).

CHAPTER II

Historical Review of Electrophoresis

Electrophoresis primarily involves the migration of macromolecules such as ionic polysaccharides, nucleic acids and proteins when subjected to an electric field. Migration is based on the net charge and size and/or shape of the macromolecule being electrophoresed and the ionic strength of the buffer solution. Other parameters determining migration include the composition of the solvent medium and the frictional coefficient of the molecules (Gaal et al., 1980).

Essentially there are three types of electrophoretic systems used today. The first is moving boundary, where there is an initially sharp boundary between the buffer and the macromolecular solution (Tiselius, 1957). The movement of the boundary determines the migration velocity of the charged macromolecules. Consequently, multiple moving boundaries depict heterogeneous solutions. However, individual components cannot be resolved into distinct zones because the density of the buffer is less than that of the macromolecular solution and diffusion of band spreading poses a problem.

The second type of electrophoresis (Tiselius,

1975) is zone electrophoresis, which involves the separation of zones, i.e., the buffer solution and the media. Methods of separation include density gradients, rotation or continuous flow of a liquid film, or the most common method, the use of a porous medium such as filter paper, cellulose acetate or various gels. Zone electrophoresis was used exclusively throughout this study.

Electromigration (Gaal et al., 1980), the third method, is a steady state or displacement electrophoresis where the width of zone does not change with time. An example of electromigration is iso-electric focusing.

Variation of Zone Electrophoresis

The first successful type of zone electrophoresis was performed using filter paper in 1950 (Gaal et al., 1980). The filter paper was composed of 96% - cellulose which was moistened in a buffer solution. Paper thickness proved to be a factor, since the conductive properties depend on the volume of buffer contained in the paper. Thin or medium-thick papers that have a dense texture resulted in straight electrophoretic fronts while thick, highly absorbing papers demonstrated indistinct fronts (Wunderly, 1954).

An important consideration of filter paper electrophoresis is electroosmosis, which may influence

fluid movement. Although electroosmosis itself should not diminish the fractionating power or induce trailing (Kunkel and Trautman, 1959), it usually makes calculations of electrophoretic mobilities somewhat difficult. Hydrostatic heads may also develop, resulting in the exudation of water at one end and drying out at the other. This phenomenon ultimately leads to the loss of homogeneity of the electrophoretic field (Wieme, 1965). Another consideration is the buffer level in each compartment which must be even in order that a siphoning effect be avoided. Ionic strength which is increased by evaporation must be guarded against for it leads to a concentration of electrolyte in the paper and poor resolution of the migrating macromolecule. Low ionic strength buffers and cooling can be used effectively to control evaporation. After electrophoresis, the paper is dried at 105-110^{o}C for 20-30 minutes and then stained (Gaal et al., 1980).

Cellulose Acetate

Cellulose acetate, which was originally prepared for filtration was introduced by Kohn (1958) as a supporting media for electrophoresis. This method is comparable to filter-paper electrophoresis. It consists of a homogeneous, microporous structure with an average pore diameter of a few microns. Cellulose acetate possesses

several advantages over filter paper. They are 1) high purity, 2) no tailing due to low absorptive properties, 3) colorless background, 4) shorter electrophoretic period and 5) decreased spreading of zones.

A disadvantage of this method is the relatively low resolution when compared to the various gels. Lipophilic dyes such as Sudan Black B are difficult to remove from the media. Cellulose acetate is used extensively in clinical laboratories for the quantitative analysis of blood proteins (Bohinski, 1979).

Polyacrylamide Gel Electrophoresis

Since the introduction of polyacrylamide gel as a supporting medium for electrophoresis (Raymond and Weintraub, 1959), it has become one of the biochemist's most important tools. The resolution of polyacrylamide gel permits the separation and characterization (charge, conformation, molecular weight) of biological macromolecules (Houtsmiller, 1969). Separations are comparable to those of starch gel due to the sieving effect (Hermans et al., 1960). Applications of polyacrylamides to disc electrophoresis have been successful (Ornstein, 1961; Davis, 1961). The separation of lipoproteins into many proteins has been achieved through the introduction of the gradient principle into this medium (Pratt and Dangerfield,

1969).

Although resolution using this medium is high, the preparation for electrophoresis is relatively laborious and the medium (acrylamide) is a potentially hazardous neural toxin when in the liquid state. Electrophoresis is carried out with a buffer that is in direct contact with the gel, and the electro-osmotic flow is virtually negligible. Due to its extreme versatility (in immunoelectrophoresis, wide pH ranges of the buffers, etc.) it has been successful in applications of various histochemical procedures (Wieme, 1965). Optically the medium is clear, and chemically it is inert and stable (Houtsmiller, 1969). Extreme care must be taken when employing this method for electrophoresis.

Starch Gel

The use of starch gel electrophoresis has become one of the major techniques in the area of protein separation. High resolution using this system is obtained by a combination of electrophoresis and differential filtration (Wieme, 1965). Since the medium possesses a fluctuating range of pore sizes, termed thermal agitation, the progress of migration of very large molecules will only be retarded but not prevented (Poulik and Smithies, 1958). Consequently, molecules that have the same charge but different molecular sizes will separate. Starch gel is

prepared by heating partially hydrolyzed starch until it forms a homogenous mass (Wieme, **1965**). The suspension suddenly becomes more viscous and the heating must be continued until the viscosity level falls. At this point the suspension is converted to a transparent solution which is then degassed in a vacuum and poured into trays (Bloemendal, **1963**).

This medium offers the researcher the versatility required for adaptation with many biological macromolecules. For example starch block electrophoresis has been adapted for the separation of lipoproteins (Kunkel and Slater, **1952**). Successful separation of lipoproteins is attributed to the fact that the absorption of proteins into the medium is almost negligible, allowing large molecules such as lipoproteins to move freely (Bloemendal, **1963**). A consequence of this method regarding lipoproteins is the relatively long staining period of **16** hours and destaining period of **6-8** days (Fine and Burstein, **1958**). Interpretation of the results of general electrophoresis using starch gels may be difficult due to the complexity of factors involved in separation (Wieme, **1965**). Some of the factors include surface distortions on the gel, variations in the type of starch, and the time and conditions of hydrolysis, all which can lead to differences in resolution

(Bier, 1959).

Agarose Gel Electrophoresis

Agarose gel was originally used for the separation of metals (Kendall, 1928) and then adapted for its use with proteins (Gordon, 1949). The breakthrough occurred when electrophoresis in agar media was extensively analyzed (Wieme, 1959).

The gel is prepared by heating agar in boiling water until the solution is clear (Wieme, 1965). The agar becomes viscous at about 38°C. Liquefaction of the media is achieved by boiling at 100°C. The structure of the agar is believed to contain at least two polysaccharides, agarose and agaropectin (Araki, 1953).

The advantages of this technique include: A) relatively low cost of media, B) high resolution, C) moderate electrophoretic period, D) non-toxic media, E) simplicity of technique, and F) transparency of media.

Taking the above factors into consideration along with the fact that agarose gel is virtually impervious to absorption of many proteins, agarose gel was chosen as the medium for electrophoretic study in this investigation since its versatility and reliability permit the adaptability necessary for acceptable results regarding the cellular system under study. Its resolving properties are

far greater than those of cellulose acetate and are comparable to acrylamide, while at the same time the preparation for electrophoresis is less hazardous and simpler than the latter.

CHAPTER III

Materials and Methods

Clones of *Didymium iridis* were secured from Dr. J. J. Yemma, Department of Biological Sciences, Youngstown State University, and O. R. Collins, Department of Botany, Berkeley, California. The clones used in this study were designated as follows: CR_5-5A^2 1-12 (Costa Rica); Pan 2-4 wt 1-12 (Panama); Pan 2-44A^8 (Panama); and Ph1A^2 (Philippines).

Culturing of cells - Myxamoeba

The original clones were maintained in milk bottle slants containing 1/2 strength (2%) corn meal agar (Table 1) that were previously inoculated with *Escherichia coli* which served as a food source for the cells. These slants were stored in an incubator set at 21°C (Yemma and Therrein, 1972; Yemma et al., 1974).

Subcultures of each clone were prepared by transferring a plug of the amoeba to sterile 100 mm x 15 mm petri plates of 1/2 strength corn meal agar previously inoculated with *E. coli*, according to the methods of Yemma and Therrien, 1972.

Plugs of arbitrary but approximate similar size from each clone were transferred to 25-30 petri plates

TABLE 1 - MEDIA

1/2 Strength Corn Meal Agar (2%)

8.5 g Difco Corn Meal Agar
8.0 g Difco Bacto-Agar
1.0 liter Distilled Water

Plasmodia Agar

15.0 g Bacto-Agar
1.0 g Bacto-Peptone
1.0 g Bacto-Lactone
1.0 liter Distilled Water

All media were mixed and boiled on a Thermolyne Type 1000 Magnetic Stirrer and then autoclaved at 121°C, 15 psi, for 15 minutes. Each sterile petri plate was filled with approximately 25 ml of media.

containing 1/2 strength corn meal agar and E. coli. Great care was exercised to maintain sterility of the clones during the transfer process.

Collection of cells - Myxamoeba

Each subculture was permitted to grow to confluency which required approximately 5-7 days or until the time prior to encystment of the myxamoeba. Twenty-five to thirty culture plates were grown and flooded in 40 ml of either of 3 buffers, Tris-Maleate, pH 8.0, Tris-Maleate, pH 7.4 or Sodium Phosphate pH 7.0 designated as buffers 1, 2 and 3 (Table 2), diluted 1:14. The diluted buffers constituted the lysate buffer. Buffers 1, 2 and 3 undiluted were used as running buffers in the investigation of specific enzymes studied. Cells were then collected in a glass centrifuge tube and counted on an American Optical 0.1 mm X 0.1 mm Hemocytometer.

The cells were concentrated by centrifugation in a Damon/IEC HNS Centrifuge for 10 minutes at 900 rpm. This procedure facilitated the collection of cells within a plug while at the same time leaving the E. coli suspended in the supernatant. After decantation, the pellet was washed twice with 20 ml of lysate buffer, centrifuged at 2,500 rpm, and the supernatant once again decanted. The cellular pellet was covered with a designated amount of lysate

TABLE 2

Lysate* Buffer	Enzymes Studied
1 Tris-Maleate Buffer pH = 8.0	Phosphoglucomutase (PGM) Isocitrate Dehydrogenase (ICDH)
2 Tris-Maleate Buffer pH = 7.4	Glutamate Oxaloacetate Transaminase (GOT) Glucose Phosphate Isomerase (PGI)
3 Sodium Phosphate Buffer pH = 7.0	Glucose-6-Phosphate Dehydrogenase (G-6-PDH) Alkaline Phosphatase Esterase D

*Lysate buffers were made by a 1:14 dilution of the running buffers,

buffer, and these were then disrupted using a Branson Sonifier 185 at a microtip setting of 5 for 15-25 seconds under ice. After centrifugation (2,500 rpm), the cell debris was discarded and the lysate was collected in Vangard #1076 2cc cryogenic tubes. The tubes were stored in a Union Carbide VHC-35 Cryogenic Tank filled with Liquid Nitrogen, at a temperature of -196°C (-320°F).

Culturing of Plasmodia

Plasmodia for this study were obtained by crossing two clones of compatible mating type on plates of 1/2 strength corn meal agar inoculated with E. coli. To ensure mating type compatibility, ten 35 mm x 10 mm petri plates of 1/2 strength corn meal agar and E. coli were used and marked for plasmodia1 formation. Plasmodia growth required approximately 7-12 days. These were then transferred to petri plates containing plasmodia agar in order to increase their size (Table 1). Fifteen plates of plasmodia were grown for buffer 1, 5 plates for buffer 2, and 5 plates for buffer 3. Plasmodia1 growth was enhanced by placing sterile oatmeal around the periphery of the plates. E. coli was not necessary for growth of this tissue and it was grown axenically.

Collection of Lysates-Plasmodia

The plates for each buffer were flooded and

washed with lysate buffer. The plasmodia were then centrifuged, sonified and collected in cryogenic tubes following the same procedure described for myxamoeba.

Protein Determination

The Lowry Procedure

Before valid quantitative electrophoresis can be performed, a protein determination for each lysate must be made. It is imperative that known amounts of protein for each lysate be placed on each well, particularly when analyzing the activity of isoenzymes. Thus the protein determination was performed according to a modified Lowry Method (1951, Table 3). A standard curve was plotted in order to determine the protein content of each lysate. It was then possible to determine the number of microliters of lysate used in each well of the electrophoretic slab.

Electrophoretic Methods

Electrophoresis of the enzymes was accomplished by placing a designated amount of lysate in well templates. Approximately 60 to 400 µg of protein were placed in each well with a Digitron SMI Electronic Pipette. A concentrated sample of E. coli, food source for the myxamoeba, was electrophoresed to determine whether or or not this organism produced variations in isoenzyme

TABLE 3

Lowry Protein Determination

Reagents:

A - 1% Na_2CO_3 in 0.1 NaOH
B - 0.5% $CuSO_4$ $5H_2O$ in 1% Sodium or Potassium Tartrate
C - 50 ml A+ 1 ml B (Prepare Daily)
D - 50 ml 2% Na_2CO_3 + 1 ml B
E - Folin reagent 1:1 with H_2O

Standards:

Dilutions of 25, 50, and 100 µg/µl of Sigma Globulin Protein Standard were used for Buffer 1 lysates; dilutions of 1.6, 8.0, and 16 µg/µl of Dade Serum Albumin were used for Buffers 2 and 3.

Procedure:

1. Mix 0.2 ml lysate and 1 ml solution C. Let stand 10 minutes at room temperature (a Clay-Adams gun was used to pipette the sample.
2. Add 0.10 ml solution E, mixing immediately.
3. After 30 minutes the absorbances were read at 750 nm on a Varion Techtron Model 635 dual wavelength Spectrophotometer.

profiles. Helena Titan Gel High Resolution Protein Agarose Gels were used throughout this study, These were commercially prepared under strict conditions of quality control, They consist of agarose in a sorbitol solution with 0.1% sodium azide and 0.02% thimersol (as a preservative). The gels accomodated up to seven samples, Two gels, one designated Amoeba and the other Plasmodia, were electrophoresed simultaneously on a Shandon Horizontal Slab Gel Electrophretic Chamber with a Shandon Southern Sae 2769 power supply. A Bromophenol tracker dye was used during each electrophoretic run to note the progress of migration, Cooling of the gels was achieved with an E-C ice water circulator and a Gorman-Rupp circulating pump.

Development of Electrophoretic Plates

After electrophoresis, each slab was placed on a Noble agar gel containing the substrate and chromogenic reagent system (charge "stain") specific for each enzyme being investigated. The following tables give the protocols of the running buffers, lysate, buffers, stains and substrates specific for each enzyme, along with the actual running conditions for the amoeba and plasmodia. Electrophoretic methods as described by Harris and Hopkinson (1976) were obtained from Dr. Richard D.

Kreutzer, Department of Biological Sciences, Youngstown State University and adapted for use on Didymium iridis. After appropriate staining, the reaction for each gel was terminated by washing them in a 5% acetic acid solution until clear, then placed in a THELCO/GCA Precision Scientific Incubator which was set at 70°C until dry.

Total Protein Electrophoresis - The Silver Stain Procedure

Total protein electrophoresis of each cell type was performed using Tris-maleate buffers at a pH of 8.0, and subsequently stained by the use of a highly sensitive silver staining procedure developed by Helena Laboratories, Beaumont, Texas. This permits the observation of total protein banding patterns.

Collection of Spores

Two plasmodia were permitted to sporulate. Electrophoresis and enzyme analysis as previously described were performed on the spores.

Quantification of Isoenzymes

Enzyme activity, per microgram of protein, was analyzed through the use of Scanning Densitometry. A Beckman Model R-112 Scanning Densitometer was used in this study. All enzymes, with the exception of esterase D, were scanned at a wavelength in the range of 550 nanometers

which was found to be the optimum peak for spectrophotometric absorption.

TABLE 4

PHOSPHOGLUCOMUTASE (PGM)

1. Running buffer: 0.1 M Tris (12.11 g T-1503), 0.1 M Maleic acid (11.62 g M-0375), 0.01 M EDTA (Na_2) (2.92 g ED2SS), 0.01 M $MgCl_2$ (2.03 g). All in 1 liter of distilled H_2O. Adjust to pH 8.0 with 40% NaOH.

2. Lysate buffer: dilute 1 part running buffer to 14 parts distilled H_2O.

3. Reaction buffer: dissolve 0.06 M Tris 0.728 g per 100 ml T1503; adjust to pH 8.0 with 50% HCl.

4. Stain: The following reagents were dissolved in 30 ml reaction buffer: 200 mg α-D-glucose-1-phosphate G-7000 or G-875, 120 mg $MgCl_2$, 15 mg MTT M-2128, 15 mg β-NADP (Na salt) N-0505, 10 mg PMS P-9625, 25 units glucose-6-phosphate dehydrogenase G-6378, 60 mg EDTA (Na_2 salt) ED2SS, 1.2 mg α-D-glucose-1.6 diphosphate G-5875.

*Dissolve 0.6 g of Noble Agar (Difco) in 30 ml of reaction buffer. While heating and stirring, allow to cool to 50°C, then add agar to stain. Pour into Mueller-Hinton culture plates.

Running Conditions: 200 volts, 60 minutes, 20 mAmp.

*Denotes that this step is carried out for each enzyme being studied, i.e., the addition of Noble agar to a specific substrate stain. The only enzyme that does not follow this procedure is Esterase D.

TABLE 5

ISOCITRATE DEHYDROGENASE (ICDH)

1. Running buffer: dissolve the following in distilled water: 0.2 M NaH_2PO_4 S-0751 (21.3 g); 0.2 M Na_2HPO_4 S-0876 (9.72 g) in 1155 ml distilled water. Adjust pH to 7.0 with 50% HCl.

2. Lysate buffer: dilute 1 part running to 14 parts distilled water.

3. Reaction buffer: dissolve 0.1 M Tris T-1503, 1.312 g Tris in 100 ml distilled water, adjust the pH to 8.0 with 50% HCl.

4. Stain: The following reagents were dissolved in 30 ml reaction buffer: 100 mg DL-Isocitric acid 11252, readjust pH to 8.0 and then add 15 mg MTT M-2128, 15 mg NADP N-0505, 10 mg PMS p-9625.

Running conditions: 200 volts, 60 minutes, 31 mAMP.

TABLE 6

GLUTOMATE OXALOACETATE TRANSAMINASE (GOT)

1. Running buffer: 0.1 ml Tris (12.11 g T-1503), 0.1 M Maleic acid (11.62 g M-0375), 0.01 M EDTA (Na_2) (2.92 g ED 2-SS), 0.01 M $MgCl_2$ (2.03 g), all in 1 liter distilled H_2O. Adjust to pH 7.4 with 40% NaOH.

2. Lysate buffer: dilute 1 part running and 14 parts distilled water.

3. Reaction buffer: dissolve 0.1 M Tris (1.312 g in 100 ml distilled water). Adjust to pH 8.0 with 50% HCl.

4. Stain: Dissolve the following in 30 ml reaction buffer:
   Substrate: 200 mg L-Aspartic acid A-9256, 75 mg α-Ketoglutaric acid (readjust pH to 8.0) K-1875, 10 mg Pyridoxal-5-phosphate p-9255.
   Stain: 75 mg fast blue BB F-3378.

Running conditions: 200 volts, 60 minutes, 24 mAMP (Run 1)
250 volts, 90 minutes, 30 mAMP (Run 2)

TABLE 7

GLUCOSE PHOSPHATE ISOMERASE (PGI)

1. Running buffer: 0.1 M Tris (12.11 g T-1503), 0.1 M Maleic acid (11.62 g M-0375), 0.01 M EDTA (Na_2) (2.92 g ED 2-SS), 0.01 M $MgCl_2$ (2.03 g) all in 1 liter of distilled H_2O. Adjust to pH 7.4 with 40% NaOH.

2. Lysate buffer: dilute 1 part running buffer and 14 parts distilled water.

3. Reaction buffer: dissolve 0.06 M Tris (0.728 g per 100 ml distilled water). Adjust to pH 8.0 with 50% HCl.

4. Stain: Dissolve the following in 30 ml reaction buffer: 20 mg Fructose-6-phosphate F-1502, 120 mg $MgCl_2$, 15 mg MTT M-2128, 15 mg β-TPN (Na salt) N-0505, 10 mg PMS P9625, 25 units Glucose-6-phosphate dehydrogenase G-6378, 60 mg EDTA (Na_2 salt) ED-2SS.

Running conditions: 200 volts, 1 hr 10 min, 20 mAMPs.

TABLE 8

GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G-6-PDH)

1. Running buffer: dissolve the following in 1155 ml of distilled water: 0.2 M NaH_2PO_4 S-0751 (21.3 g), 0.2 M Na_2HPO_4 S-0876 (9.72 g). Adjust to pH 7.0 with 50% HCl.

2. Lysate buffer: dilute 1 part running buffer to 14 parts distilled water.

3. Reaction buffer: dissolve 0.06 M Tris (0.728 g in 100 ml distilled water). Adjust to pH 8.0 with 50% HCl.

4. Stain: Dissolve the following in 30 ml of reaction buffer: 75 mg D-Glucose-6-phosphate G-7375, 15 Mg MTT M-2128, 15 mg β-TPN (Na salt) N-0505, 10 mg PMS P-9625.

Running conditions: 200 volts, 40 min, 28 mAMP (Run 1)
200 volts, 2 hrs, 28 mAMP (Run 2)

TABLE 9

ALKALINE PHOSPHATASE

1. Running buffer: 0.15 M Citric acid (Na_3) (38.7 g C-7254), 0.24 M NaH_2PO_4 (28.8 g S-0751). Adjust to pH 6.3 with 40% NaOH. Dissolve in 1 liter of distilled water.

2. Lysate buffer: dilute 1 part running buffer to 14 parts distilled water.

3. Reaction buffer: 0.06 M Boric acid (0.37 g B-0252) in 100 ml of distilled water. Adjust to pH 8.3 with 40% NaOH.

4. Stain: Dissolve the following in 30 ml reaction buffer: 75 mg α-naphthyl phosphate N-1132, 100 mg $MgSO_4$ M-1880, 30 mg fast blue BB F-3378.

Running conditions: 200 volts, 50 min, 38 mAMPs.

TABLE 10

ESTERASE D

1. Running buffer: 0.1 M Tris (12.11 g T-1503), 0.1 M Maleic acid (11.62 g M-0375), 0.01 M EDTA (Na_2) (2.92 ED2-SS), 0.01 M $MgCl_2$ (2.03 g), 0.15 M NaOH (6.0 g). Dissolve in 1 liter of distilled water. Adjust pH to 7.4.

2. Reaction buffer: 0.41 g Sodium acetate anhydrous (0.05 M). Adjust to pH 6.5 with 1% acetic acid (dissolve first in 100 ml of distilled water).

3. Stain: dissolve 4 mg of 4-Methylumbelliferyl acetate (M-0883) in 300 ml of acetone and then add to 10 ml of reaction buffer.

4. Application: Soak stain on Whatman 3 mm filter paper and apply to electrophoretic plate. Incubate at room temperature for 5 minutes. Read plate using long wavelength ultraviolet light and mark bands.

Running conditions: 200 volts, 1 hr, 31 mAMP.

CHAPTER IV

Results

The results of this study are presented in tabular form and graphically as electropherograms of each enzyme. The diagrams represent total protein applied to the gel, and relative activity (content), and migration patterns of enzymes. Where applicable, the enzyme polymorphism for myxamoebal clones and plasmodia are presented. Densitometric scan patterns were used to quantitatively illustrate the relative protein content or activity of each isoenzyme band. An electropherogram illustrating total protein for the myxamoeba and plasmodia is also included.

Relative cell counts used for the electrophoretic myxamoebal sample as determined by a hand counter and hemocytometer are illustrated by Table 11, while Table 12 is indicative of the mating type compatibility for the specified clones regarding their ability to form plasmodia. Each separate cross was made on 10 petri plates of 1/2 strength corn meal agar, and scored for plasmodia1 formation. A frequency of 7 out of 10 crosses was considered an acceptable value regarding compatibility. Note that no plasmodia were formed in cases where a clone was crossed against itself as expected, indicating that

compatibility regarding the genetic mating locus requires clones possessing a mating allele other than one that is common.

Protein Assay - The Lowry Method

The results of the standard curves generated using the Lowry method of Protein Determination are illustrated in Figure 2 and Tables **13** and **14**.

Figure 2A represents the actual standard curve plotted for the less concentrated cell lysate in buffers 2 and **3**. Dilutions of **1.6**, 8 and **16** µg/µl of Dade Albumin Protein Standard were plotted against absorbance in order to measure the concentration of each sample. The curves in each case were computer generated. The amount of protein in ul that was loaded on the experimental gel to give an electrophoretic pattern was then determined. The standard curve plotted for the more concentrated lysate used in this study is reflected in Figure 2B.

Dilutions of **25**, **50** and **100** µg/µl of Sigma Globulin Protein Standard **15506** were calibrated against relative absorbances of each clone and its cross. Similarly µl of protein to be loaded on the experimental gel was determined from the graph.

Tables **13** and **14** indicate the results obtained from the graph for buffers **1**, 2 and **3**. The number of µg of

TABLE 11

Relative Cell Counts

Clones	Lysate Buffer 1*	Lysate Buffer 2	Lysate Buffer 3
1. CR_5-5A^2 1-12	44,400,000 cells per 0.4 ml lysate	40,720,000 cells per 3 ml lysate	42,042,000 cells per 3 ml lysate
2. Pan 2-4 wt 1-12	72,400,000 cells per 0.6 ml lysate	69,000,000 cells per 3 ml lysate	84,924,000 cells per 3 ml lysate
3. Pan 2-44A^8	22,500,000 cells per 0.2 ml lysate	42,500,000 cells per 3 ml lysate	43,320,000 cells per 3 ml lysate
4. PhlA^2	38,200,000 cells per 0.4 ml lysate	43,585,000 cells per 3 ml lysate	42,685,000 cells per 3 ml lysate

"Denotes a highly concentrated form of each lysate.

TABLE 12

Compatability of Mating Types

	CR5-5A^{2} 1-12	Pan 2-4 wt 1-12	Pan 2-44A^{8}	PH1A^{2}
CR5-5A^{2} 1-12	No* Plasmodia	10/10 Plasmodia	9/10 Plasmodia	10/10 Plasmodia
Pan 2-4 wt 1-12	8/10	No Plasmodia	10/10 Plasmodia	10/10 Plasmodia
Pan 2-44A^{8}	7/10 Plasmodia	9/10 Plasmodia	No Plasmodia	8/10 Plasmodia
Ph1A^{2}	10/10 Plasmodia	9/10 Plasmodia	8/10 Plasmodia	No Plasmodia

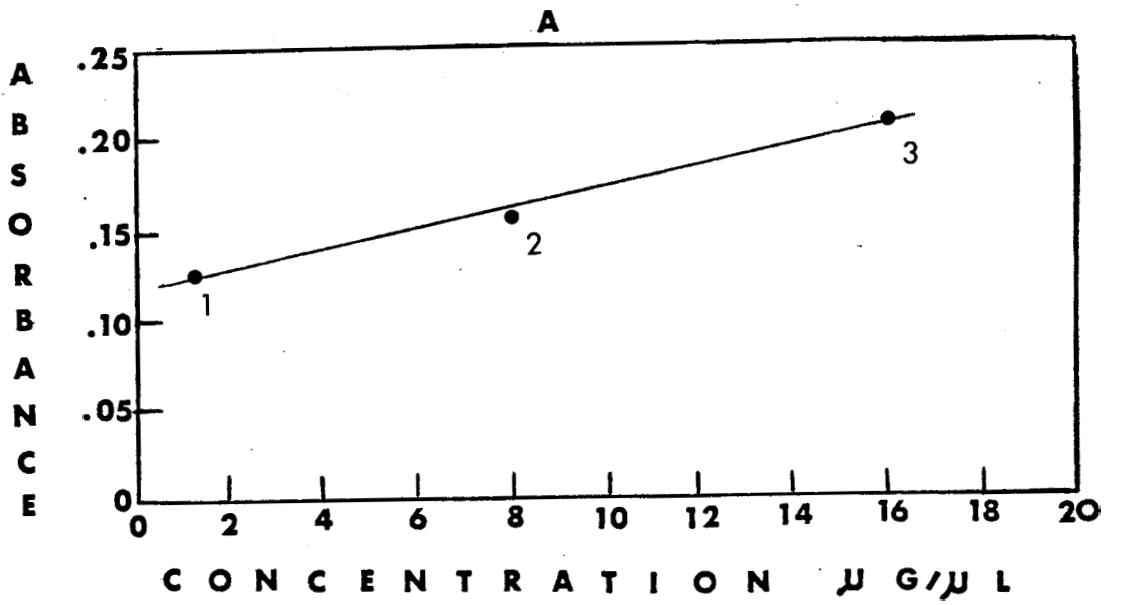
*Denotes number of crosses out of 10 that yielded plasmodia

Figure 2

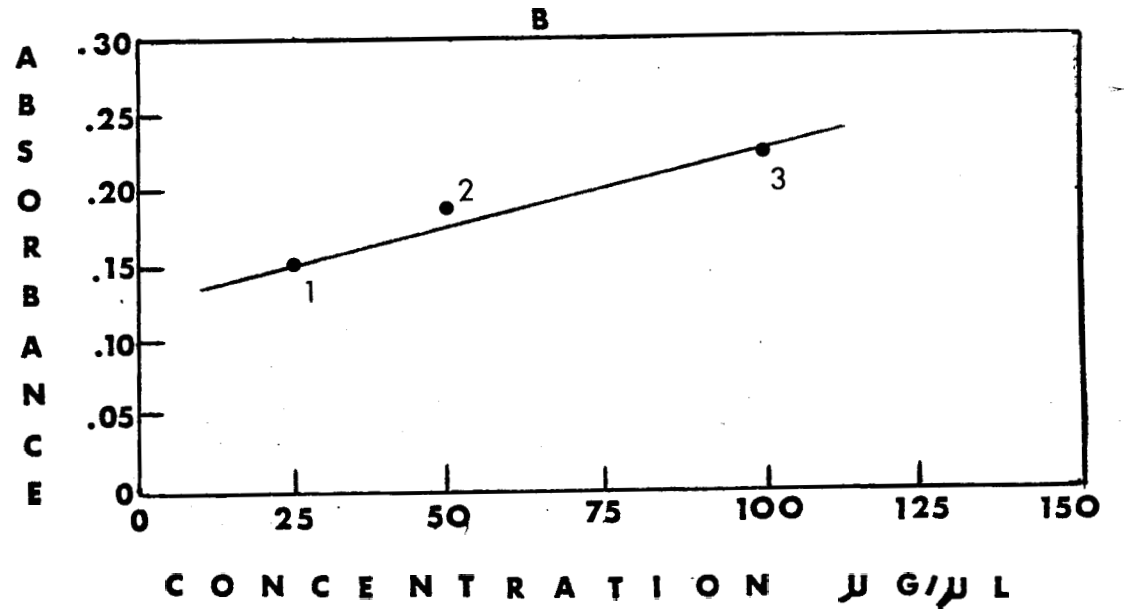
Standard curve for protein determination for:

A) Lysate Buffers 2 and 3; and

B) **Lysate Buffer 1**



Point	Standard Deviation	Standard Error
1	.0026	.0015
2	.014	.008
3	.0067	.0038



Point	Standard Deviation	Standard Error
1	.0026	.0015
2	.0168	.010
3	.0066	.0038

TABLE 13

Protein Determination – Standard Curve

Lysate Buffer 1 – Amoeba

Clone	Average Absorbance	µg of Protein	Load on Gel (µl)
CR5–5A^{2} 1-12	0.212	200	2.1
Pan 2-4 wt 1-12	0.228	200	1.8
Pan 2–44A^{8}	0.207	200	2.3
PhlA^{2}	0.203	200	2.4
	Lysate Buffer 2 - Amoeba		
CR5-5A^{2} 1-12	0.197	30	2.3
Pan 2-4 wt 1-12	0.205	30	2.1
Pan 2–44A^{8}	0.166	30	3.6
PhlA^{2}	0.176	30	3.0
	Lysate Buffer 3 - Amoeba		
CR5-5A^{2} 1-12	0.189	30	2.5
Pan 2-4 wt 1-12	0.212	30	1.9
Pan 2–44A^{8}	0.190	30	2.5
PhlA^{2}	0.176	30	3.0

TABLE 14

Protein Determination - Standard Curve

Lysate Buffer 1 - Plasmodia

Cross	Average Absorbance	µg of Protein	Load on Gel (µl)
Pan 2-4 wt 1-12 X $PhlA^2$	0.208	200	2.2
$CR5-5A^2$ X $PhlA^2$	0.233	200	1.7
Pan 2-4 wt 1-12 X $CR5-5A^2$ 1-12	0.194	200	2.8
$PhlA^2$ X Pan $2-44A^8$	0.247	200	1.5
Pan $2-44A^8$ X $CR5-5A^2$ 1-12	0.225	200	1.8
Pan 2-4 wt 1-12 X Pan $2-44A^8$	0.191	200	2.9

Lysate Buffer 2 - Plasmodia

Pan 2-4 wt 1-12 X $PhlA^2$	0.208	30	2.0
$CR5-5A^2$ X $PhlA^2$	0.210	30	2.0
Pan 2-4 wt 1-12 X $CR5-5A^2$ 1-12	0.205	30	2.1
$PhlA^2$ X Pan $2-44A^8$	0.194	30	2.4
Pan $2-44A^8$ X $CR5-5A^2$ 1-12	0.184	30	2.7
Pan 2-4 wt 1-12 X Pan 2-44 A^8	0.182	30	2.8

Lysate Buffer 3 - Plasmodia

Pan 2-4 wt 1-12 X $PhlA^2$	0.255	30	1.4
$CR5-5A^2$ X $PhlA^2$	0.236	30	1.6
Pan 2-4 wt 1-12 X $CR5-5A^2$ 1-12	0.237	30	1.5
$PhlA^2$ X Pan $2-44A^8$	0.191	30	2.4
Pan $2-44A^8$ X $CR5-5A^2$ 1-12	0.278	30	1.2
Pan 2-4 wt 1-12 X Pan $2-44A^8$	0.238	30	1.5

protein corresponds with the ul sample loaded on the gel. This provided for the application of the same amount of protein and thus the standardization of sample concentration in each well of the gel.

Control Samples

Electrophoresis of the concentrated sample of E. coli revealed no observable isoenzyme patterns. The use of sterile oatmeal as the plasmodia1 food source yielded no detectable changes in isoenzyme patterns as well.

Interpretation of Electropherograms

Diagrams representing the banding patterns of various enzymes throughout the cell cycle are included. In each case, with the exception of the silver stained protein pattern and Esterase-D, a horizontal densitometic scan of similar and major bands was done and compared for each specimen.

The scanning densitometer indicates relative amounts of protein by calculating the area under the curve. To the right of each diagram is the printout of protein concentrations of each scan which is in relative percentages of protein (the extreme right column), and corresponding numerical values of Relative Protein (R.P., the left column). Total protein amounts designated by a "P" were calculated by the summation of microgram amounts of protein

that was placed on the gel for each specimen. The first relative protein and percentage values, i.e. the values closest to the Total Protein value designated by "P" corresponds to the first clone or cross, the second value up corresponds to the second clone or cross, etc. The relative amount of enzyme protein was then calculated for each band. On the bottom right hand corner of each diagram is a key representing the relative intensity of the bands as they appeared on the gel. All lysates were placed on the origin of initial application and migrated appropriately from the origin, either in the positive or negative direction as the case may be.

For the enzymes Phosphoglucomutase (PGM), (Figures 3 and 4), and Glucose Phosphate Isomerase (PGI), (Figures 7 and 8), all which show two bands, vertical scan patterns are presented showing relative enzyme content and activity within a particular specimen.

Analysis of Electropherograms - Isoenzyme Patterns

Phosphoglucomutase (PGM)

Analysis of this enzyme (Fig. 3) shows the presence of two bands for nearly all the myxamoeba specimens with similar but varying intensities. Pan 2-44A^{8} appears to have the lowest enzyme activity (intensity) as well as the greatest band difference among the clones represented

here.

The results of the plasmodia (Fig. 4) show similar banding patterns and somewhat lower activity when compared to the myxamoeba with the exception of crosses Ph1A^2 X Pan 2-44A^8 and Pan 2 44A^8 X CR5-5A^2 1-12 having only single bands. Pan 2-4 wt 1-12 X CR5-5A^2 1-12 has the highest activity as indicated by its numerical protein value (870). Pan 2-44A^8 X CR5-5A^2 1-12 has the lowest activity among the specimens. Migration of bands was anodal and ranged from one quarter to one half the distance of the gel, depending on the band being observed. In each case, the more active bands were closer to the cathode. Note the vertical scan patterns representing multiple bands with a specimen.

Isocitrate Dehydrogenase (ICDH)

The results of ICDH are illustrated in Fig. 5A and 5B. ICDH, a Kreb's cycle enzyme demonstrated significantly higher activity for the plasmodia when compared to the Myxamoeba, as shown by the densitometer scans and intensity levels. Plasmodia1 crosses Pan 2-4 wt 1-12 X PhlA^2, CR5-5A^2 1-12 X PhlA^2 and Pan 2-4 wt 1-12 X CR5-5A^2 1-12 showed significantly increased levels of activity over the other specimens. Migration of the enzymes was approximately one quarter the distance of the gel.

Glutamate Oxaloacetate Transaminase (GOT)

The activity of this enzyme for the myxamoeba (Fig. 6A) was relatively lower and consistent among the clones when compared to the plasmodia. Plasmodia activity (Fig. 6B) was equal in two cases and higher than the myxamoeba in the remaining four cases. Especially interesting regarding this enzyme is the mode of migration. GOT is the only enzyme examined that resulted in a cathodal migration, meaning the net charge of the isoenzyme was positive. It should also be noted that greater activity among the plasmodia involved either Pan 2-4 wt 1-12 or a Pan 2-44A^{8} as parents.

Glucose Phosphate Isomerase (PGI)

This enzyme showed extremely high activity among the specimens, In every case for the myxamoeba (Fig. 7) two bands were found. To the bottom of the diagram is a vertical scan within each specimen. Protein numerical values are given, with the first and smallest number corresponding to the lowest peak and the highest number corresponding to the highest peak (and the most intense band).

Figure 8, representing the plasmodia, shows relatively the same activity when compared to in activity to the myxamoeba. Notice crosses Ph1A^{2} X Pan 2-44A^{8} and Pan

Figure 3.

Electropherogram Representing Phosphoglucornutase (PGM) for Myxamoeba.

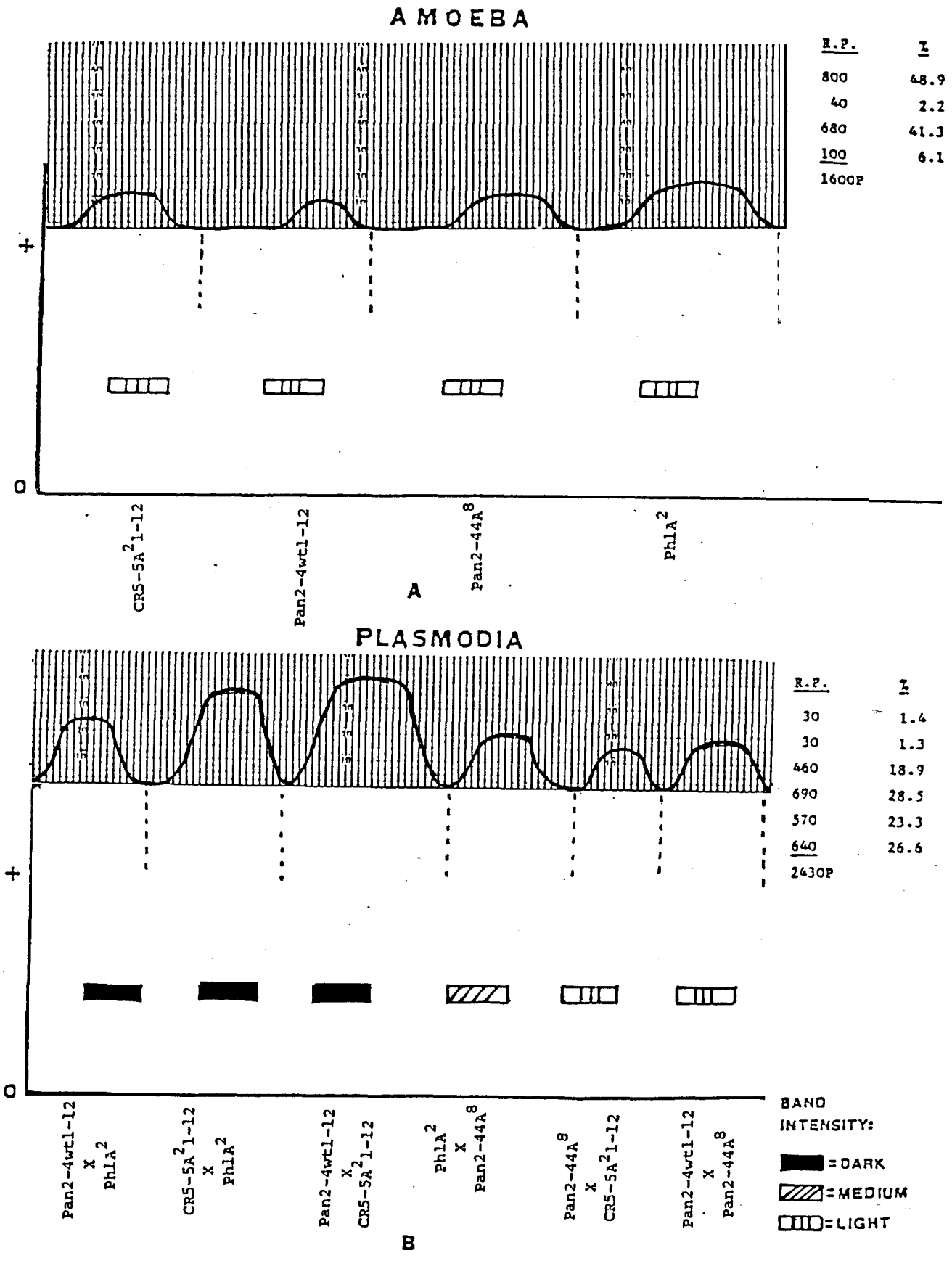
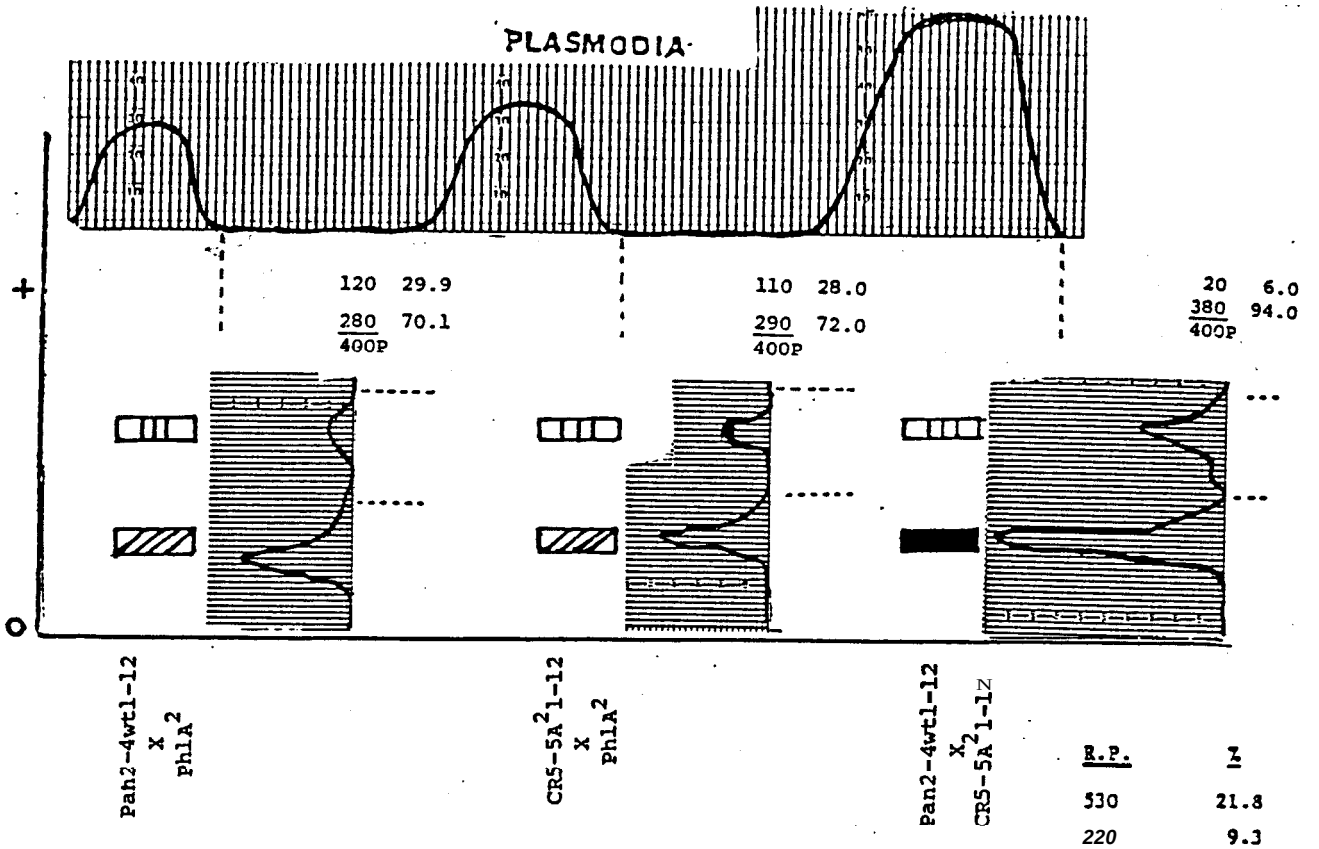

AMOEBA
R.P.
%
800
48.9
40
2.2
680
41.3
100
6.1
1600P
+
0
CR5-5A²1-12
Pan2-4wt1-12
Pan2-44A⁸
PhlA²
A
PLASMODIA
R.P.
%
30
1.4
30
1.3
460
18.9
690
28.5
570
23.3
640
26.6
2430P
+
0
Pan2-4wt1-12 X PhlA²
CR5-5A²1-12 X PhlA²
Pan2-4wt1-12 X CR5-5A²1-12
PhlA² X Pan2-44A⁸
Pan2-44A⁸ X CR5-5A²1-12
Pan2-4wt1-12 X Pan2-44A⁸
B
BAND INTENSITY:
= DARK
= MEDIUM
= LIGHT

Figure 4.

Electropherogram Representing Phosphoglucomutase (PGM) for Plasmodia.



R.P.	%
530	21.8
220	9.3
230	9.4
870	35.7
270	11.2
300	12.4
2430P	

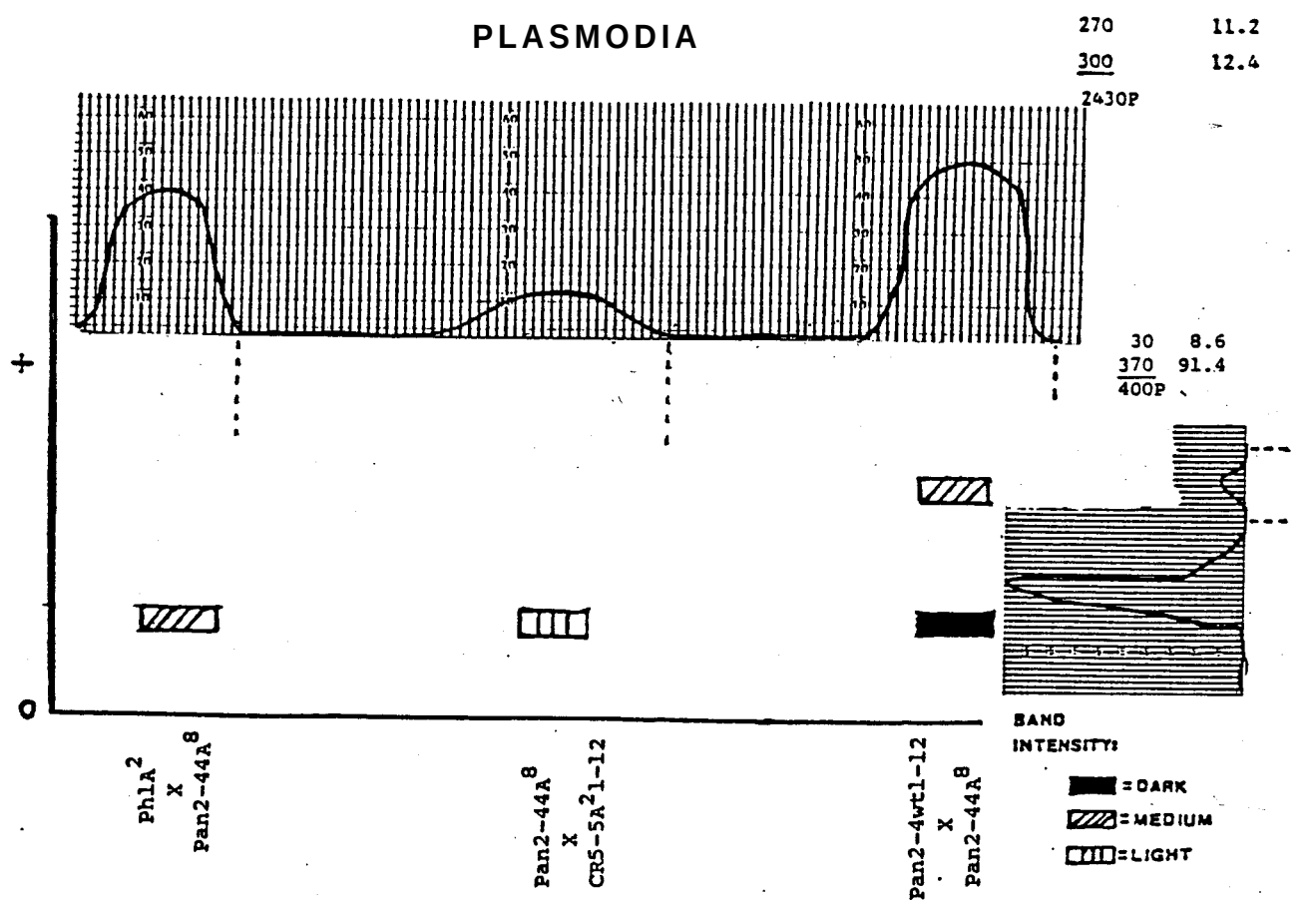


Figure 5.

Electropherogram Representing Isocitrate Dehydrogenase (ICDH) for:

(A) Myxamoeba

(B) Plasmodia

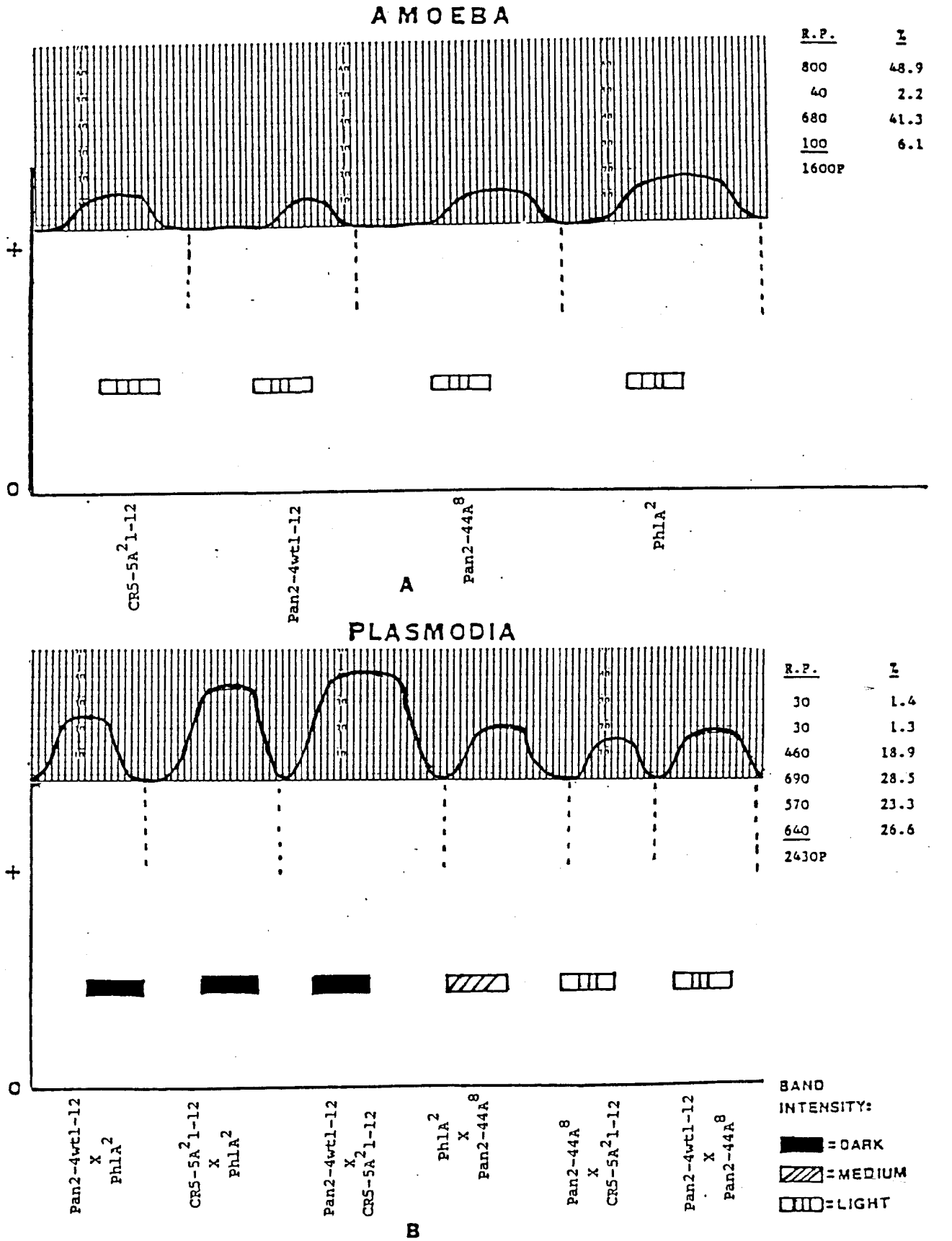
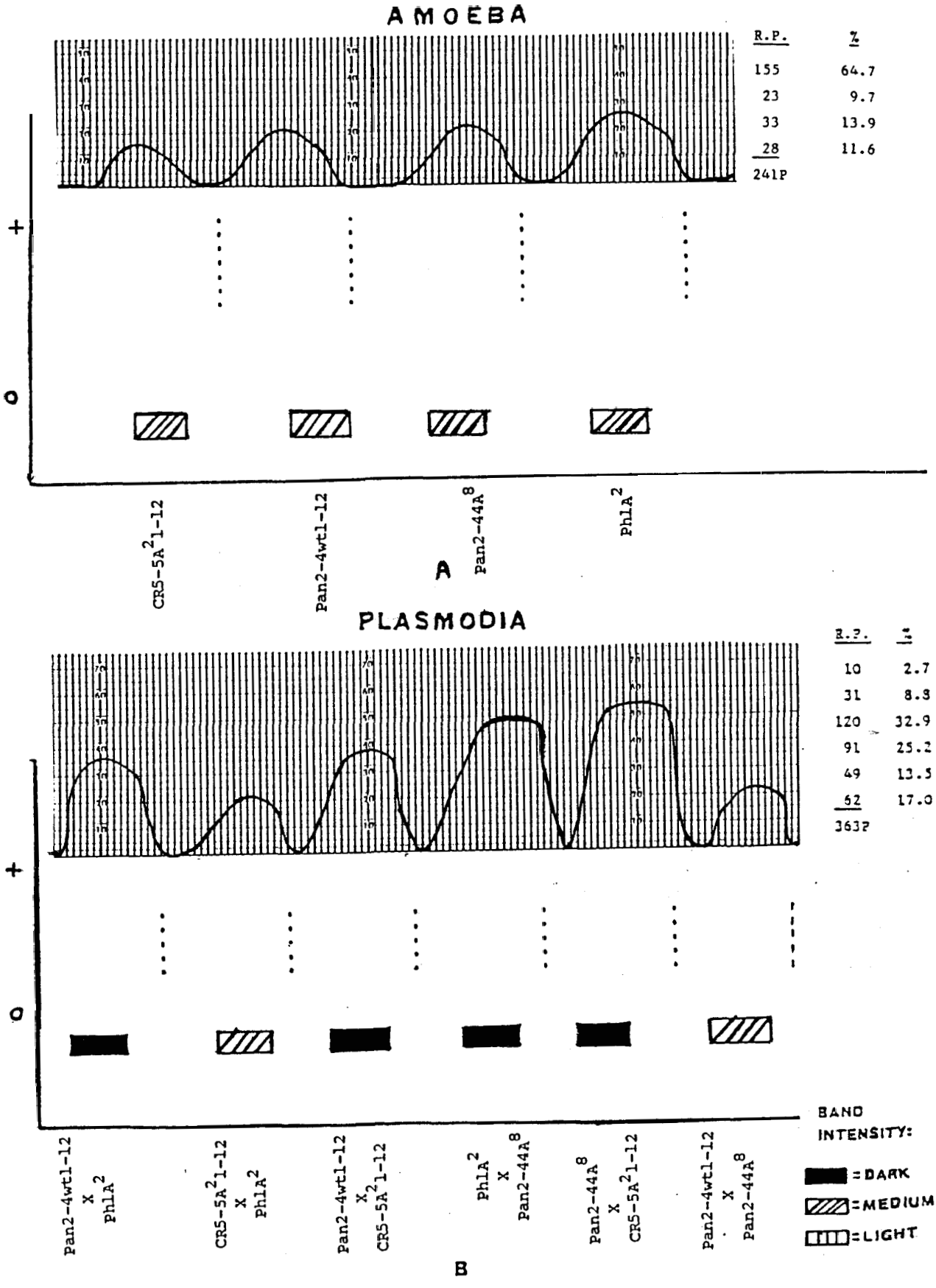

AMOEBA
R.P.
%
800
48.9
40
2.2
680
41.3
100
6.1
1600P
+
0
CR5-5A²1-12
Pan2-4wt1-12
Pan2-44A⁸
PhlA²
A
PLASMODIA
R.P.
%
30
1.4
30
1.3
460
18.9
690
28.5
570
23.3
640
26.6
2430P
+
0
Pan2-4wt1-12 X PhlA²
CR5-5A²1-12 X PhlA²
Pan2-4wt1-12 X CR5-5A²1-12
PhlA² X Pan2-44A⁸
Pan2-44A⁸ X CR5-5A²1-12
Pan2-4wt1-12 X Pan2-44A⁸
BAND INTENSITY:
= DARK
= MEDIUM
= LIGHT
B

Figure 6.

Electropherogram Representing Glutamate Oxaloacetate Transaminase (GOT) for:

(A) Myxamoeba

(B) Plasmodia


AMOEBA
R.P.
%
155
64.7
23
9.7
33
13.9
28
11.6
241P
+
0
CR5-5A21-12
Pan2-4wt1-12
Pan2-44A8
PhlA2
A
PLASMODIA
R.P.
%
10
2.7
31
8.3
120
32.9
91
25.2
49
13.5
62
17.0
363P
+
0
Pan2-4wt1-12 X PhlA2
CR5-5A21-12 X PhlA2
Pan2-4wt1-12 X CR5-5A21-12
PhlA2 X Pan2-44A8
Pan2-44A8 X CR5-5A21-12
Pan2-4wt1-12 X Pan2-44A8
BAND INTENSITY:
=DARK
=MEDIUM
=LIGHT
B

2-4 wt 1-12 X Pan 2-44A^{8} where only single bands appeared.

Migration of the bands for both the myxamoeba and plasmodia were uniform, with the most intense band migrating closer to the anode. This was just the opposite to PGM. Migration distances were one quarter to one half the gel, depending on the band being observed.

Since PGI proved to be an extremely active enzyme, it was chosen for analysis of spores (Fig. 9). Nine sporangia were taken from the designated crosses and sonified in 200 µl of membrane buffer. Designated amounts of the homogenate were placed on the well. As Fig. 9 indicates, Pan 2-4 wt 1-12 X CR5-5A^{2} 1-12 has a significantly greater activity than CR5-5A^{2} 1-12 X Phl-A^{2}.

Glucose-6-Phosphate Dehydrogenase (G-6-PDH)

The myxamoeba results are represented by Fig. 10A. Pan 2-4 wt 1-12 and PH1A^{2} have the greatest activity (intensity), while Pan 2-44A^{8} has the lowest. Overall the myxamoeba (Fig. 10B) shows slightly greater enzyme activity than the plasmodia, ranging from dark to light. Crosses CR5-5^{A} 1-12 X Ph1A^{2} and Pan 2-4 wt 1-12 X CR5-5A^{2} 1-12 showed the highest activity, Pan 2-4 wt 1-12 X Ph1A, PhlA^{2} X Pan 2-44A^{8}, Pan 2-44A^{8} X CR5-5A^{2} 1-12 intermediate,

and Pan 2-4 wt 1-12 X Pan 2-44A^{8} the lowest activity.

Net migration of this enzyme was anodal. This enzyme demonstrated limited migration out of all the enzymes tested, which was due to its great difference in mass to charge ratio.

Alkaline Phosphatase

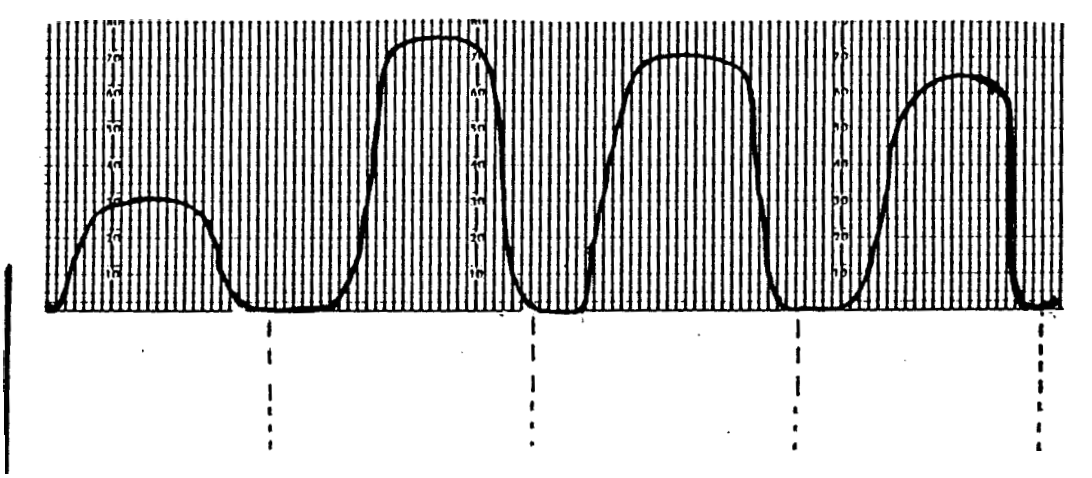
This enzyme demonstrated relatively lower activity for the myxamoeba (Fig. 11A) when compared to the plasmodia (Fig. 11B). The bands however are considerably wider for the myxamoeba. Crosses CR5-5A^{2} 1-12 X PhlA^{2}, Pan 2-4 wt 1-12 X CR5-5A^{2} 1-12, and Pan 2-4 wt 1-12 X Pan 2-44A^{8} of the plasmodia had the highest activity, Pan 2-4 wt 1-12 X PhlA^{2} and PhlA^{2} X Pan 2-44A^{8} intermediate, and Pan 2-44A^{8} X CR5-5A^{2} 1-12 the least. This enzyme demonstrated by far the greatest amount of migration which was anodal.

Because of this enzyme's high activity and extended migration distances, it was chosen for analysis of the spores (Fig. 12). The same cellular material in the form of homogenate was used as previously described for PGI spore analysis. The activity varied and once again is represented as heavy, light or intermediate intensities. Migration distances were comparable to the amoeba and plasmodia.

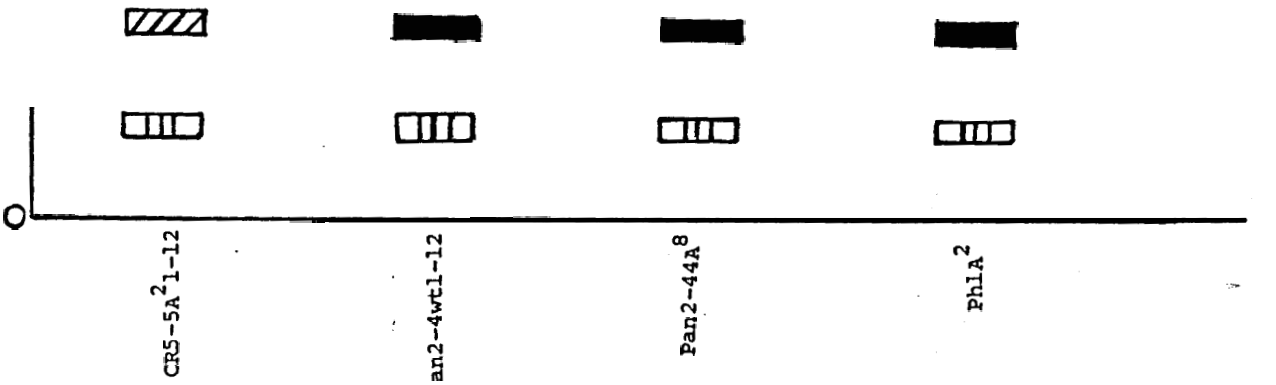
Figure 7.

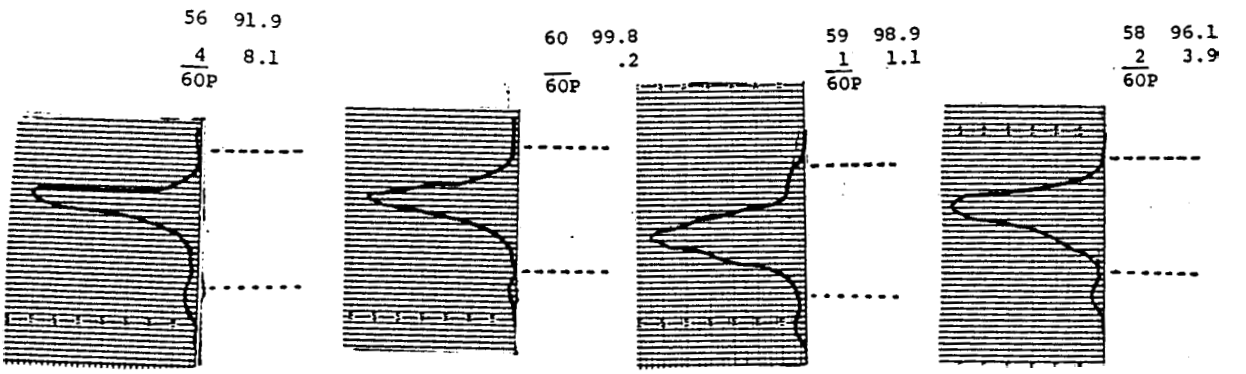
Electropherograrn Representing Glucose Phosphate Isomerase (PGI) for Myxamoeba.

AMOEBA



R.P.	%
58	24.2
76	31.9
78	32.3
28	11.6
241P	





BAND INTENSITY: = DARK

= MEDIUM

= LIGHT

Figure 8.

Electropherogram Representing Glucose Phosphate Isomerase (PGI) for Plasmodia.

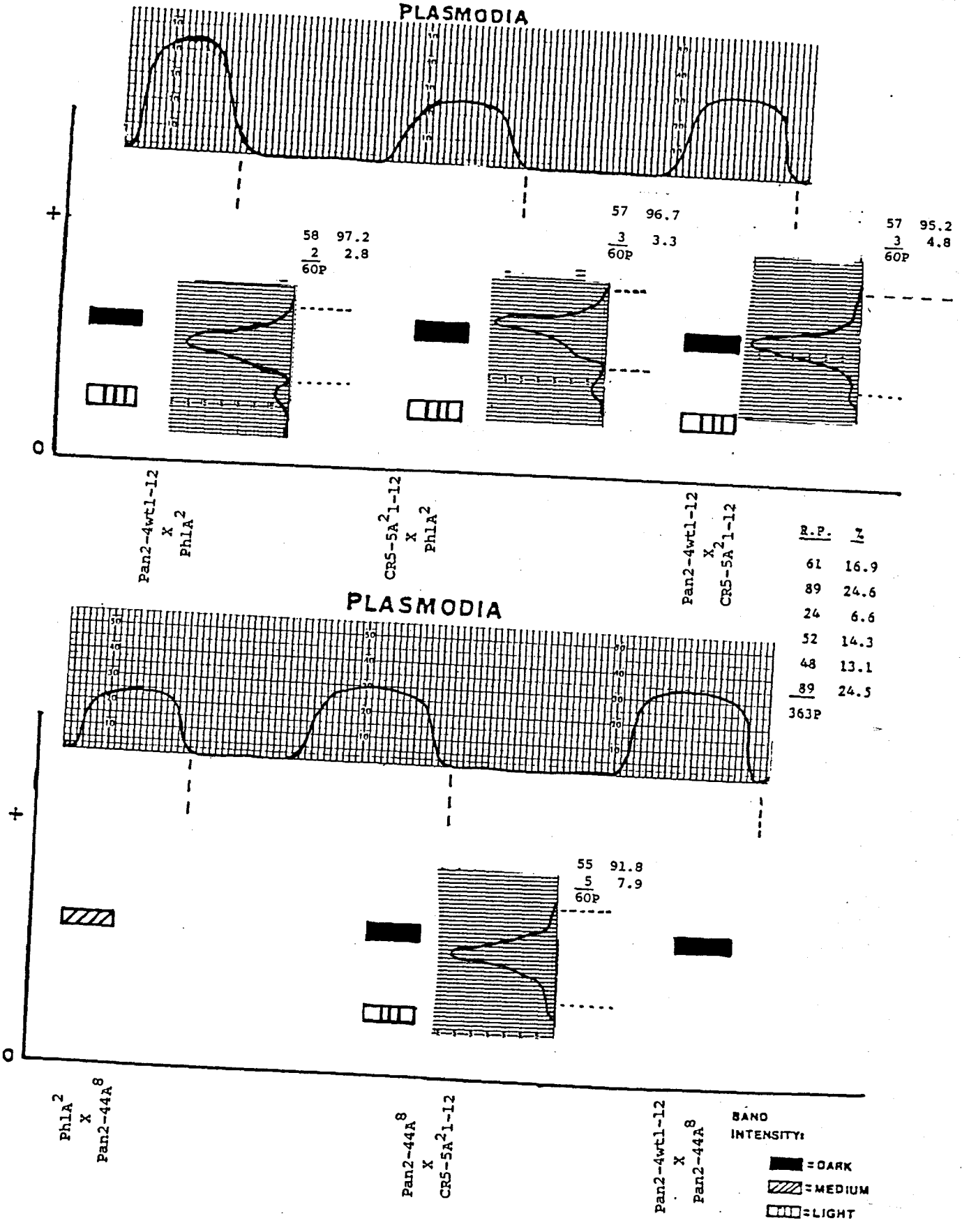
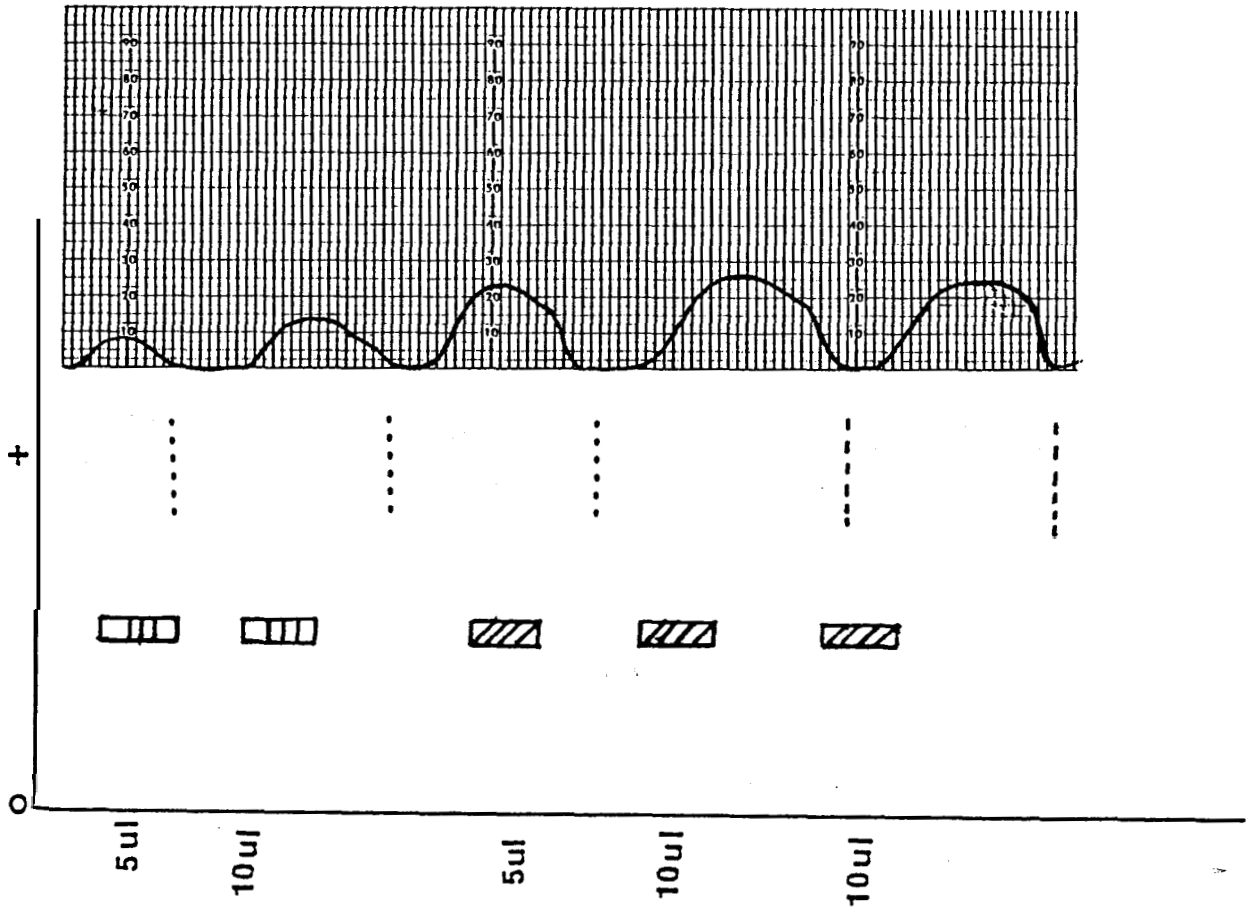

PLASMODIA
+
0
58 97.2
2 2.8
60P
57 96.7
3 3.3
60P
57 95.2
3 4.8
60P
Pan2-4wt1-12 X PhlA2
CR5-5A21-12 X PhlA2
Pan2-4wt1-12 X2 CR5-5A21-12
R.P. %
61 16.9
89 24.6
24 6.6
52 14.3
48 13.1
89 24.5
363P
PLASMODIA
+
0
55 91.8
5 7.9
60P
PhlA2 X Pan2-44A8
Pan2-44A8 X CR5-5A21-12
Pan2-4wt1-12 X Pan2-44A8
BAND INTENSITY:
= DARK
= MEDIUM
= LIGHT

Figure 9.

Electropherogram Representing Glucose Phosphate Isomerase for Spores.

SPORES



CR5-5A^{2}1-12
X
Ph1A^{2}

Pan2-4wt1-12
X$_{2}$
CR5-5A^{2}1-12

BAND
INTENSITY:

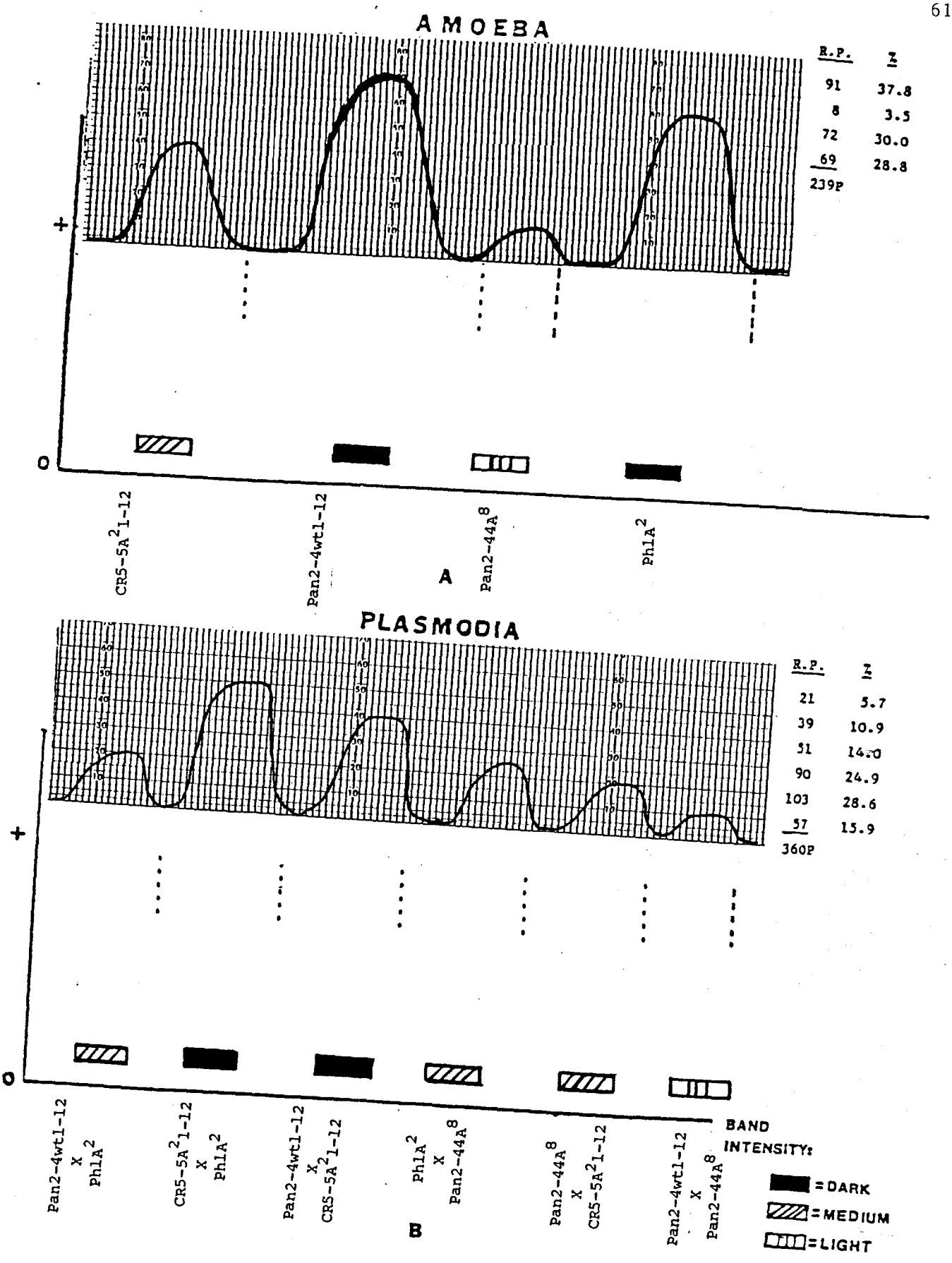
= DARK
= MEDIUM
= LIGHT

Figure 10.

Electropherogram representing Glucose-6-Phosphate Dehydrogenase (G-6-PDH) for:

(A) Myxamoeba

(B) Plasmodia


AMOEBA
R.P.
%
91
37.8
8
3.5
72
30.0
69
28.8
239P
+
0
CR5-5A²1-12
Pan2-4wt1-12
Pan2-44A⁸
PhlA²
A
PLASMODIA
R.P.
%
21
5.7
39
10.9
51
14.0
90
24.9
103
28.6
57
15.9
360P
+
0
Pan2-4wt1-12 X PhlA²
CR5-5A²1-12 X PhlA²
Pan2-4wt1-12 X CR5-5A²1-12
PhlA² X Pan2-44A⁸
Pan2-44A⁸ X CR5-5A²1-12
Pan2-4wt1-12 X Pan2-44A⁸
B
BAND INTENSITY:
= DARK
= MEDIUM
= LIGHT

Esterase D

Figure 13 illustrates the results of Esterase D for the myxamoeba. Due to the intensity produced by the fluorescent nature of the bands and residual interband staining, it was beyond the Scanning Densitometer's range and capability to obtain adequate scan patterns. However, accurate visual observations were possible. The bands are single, relatively wide, and dense in most cases, Migration was anodal. CR5-5A^2 1-12 and PhlA^2 demonstrated the greatest activity, while Pan 2-4 wt 1-12 showed the least,

Figure 14 shows interesting results concerning the plasmodia crosses Pan 2-4 wt 1-12 X CR5-5A^2 and Pan 2-44A^8 X CR5-5A^2 1-12 where CR5-5A^2 1-12 is a common parent and demonstrated the greatest activity while crosses Pan 2-4 wt 1-12 X PhlA^2 and PhlA^2 X Pan 2-44A^8 where PH1A^2 is a parent, demonstrated the least activity. Note the multiple bands for crosses Ph1A^2 X Pan 2-44A^8 and Pan 2-4 wt 1-12 X Pan 2-44A^8.

Modes of migration were similar for both the myxamoeba and plasmodia resulting in a distance of approximately one half the distance of the gel.

Silver Stain - Total Protein

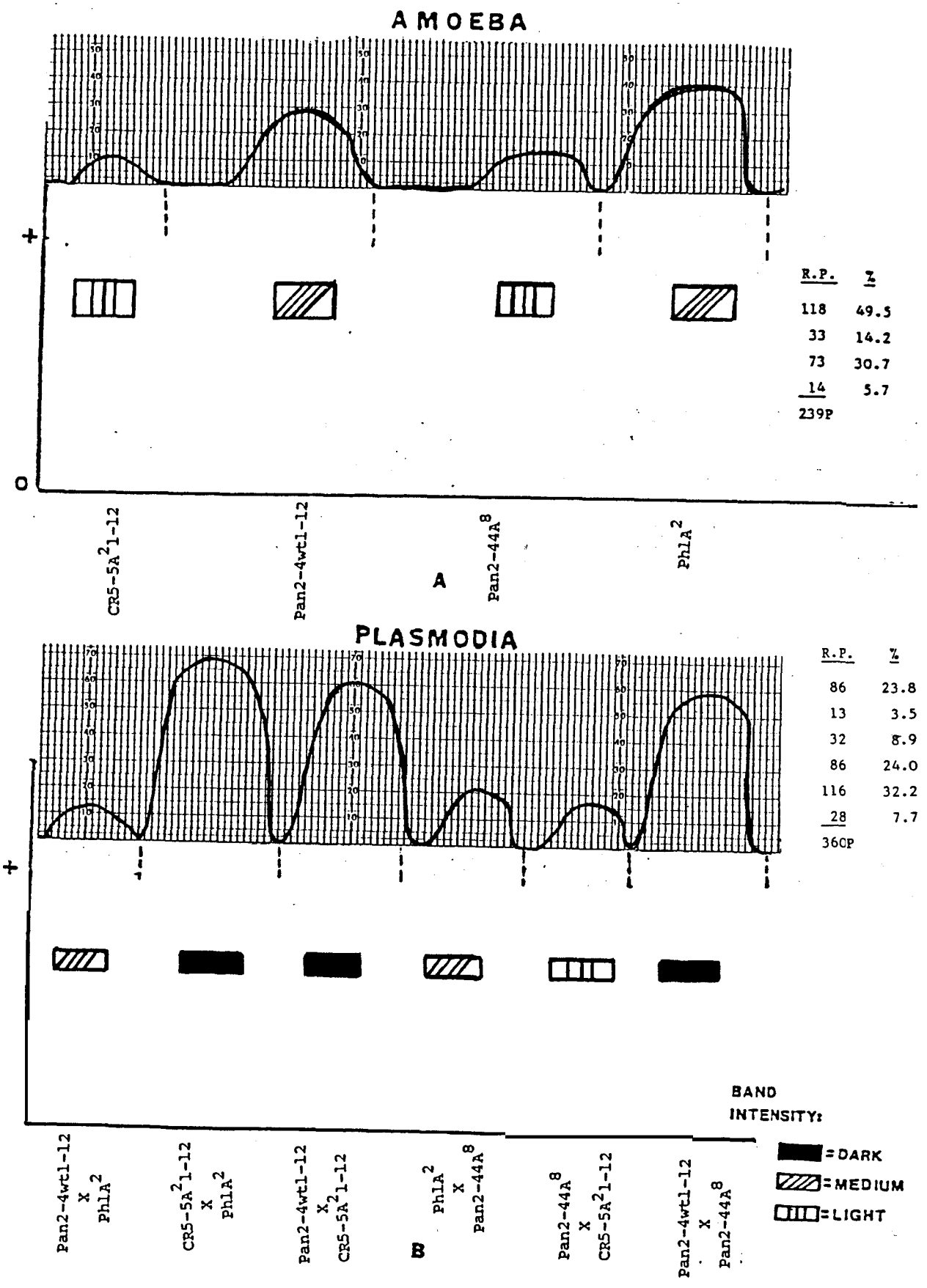
Total protein analysis for the myxamoeba (Fig. 15A) shows the majority of the bands being medium to light

Figure 11.

Electropherogram Representing Alkaline Phosphatase for

(A) Myxamoeba

(B) Plasmodia


AMOEBA
+
0
R.P. %
118 49.5
33 14.2
73 30.7
14 5.7
239P
CR5-5A²1-12
Pan2-4wt1-12
Pan2-44A⁸
PhlA²
A
PLASMODIA
+
R.P. %
86 23.8
13 3.5
32 8.9
86 24.0
116 32.2
28 7.7
360P
BAND INTENSITY:
=DARK
=MEDIUM
=LIGHT
Pan2-4wt1-12 X PhlA²
CR5-5A²1-12 X PhlA²
Pan2-4wt1-12 X CR5-5A²1-12
PhlA² X Pan2-44A⁸
Pan2-44A⁸ X CR5-5A²1-12
Pan2-4wt1-12 X Pan2-44A⁸
B

intensity, with the exception of PhlA^{2}.

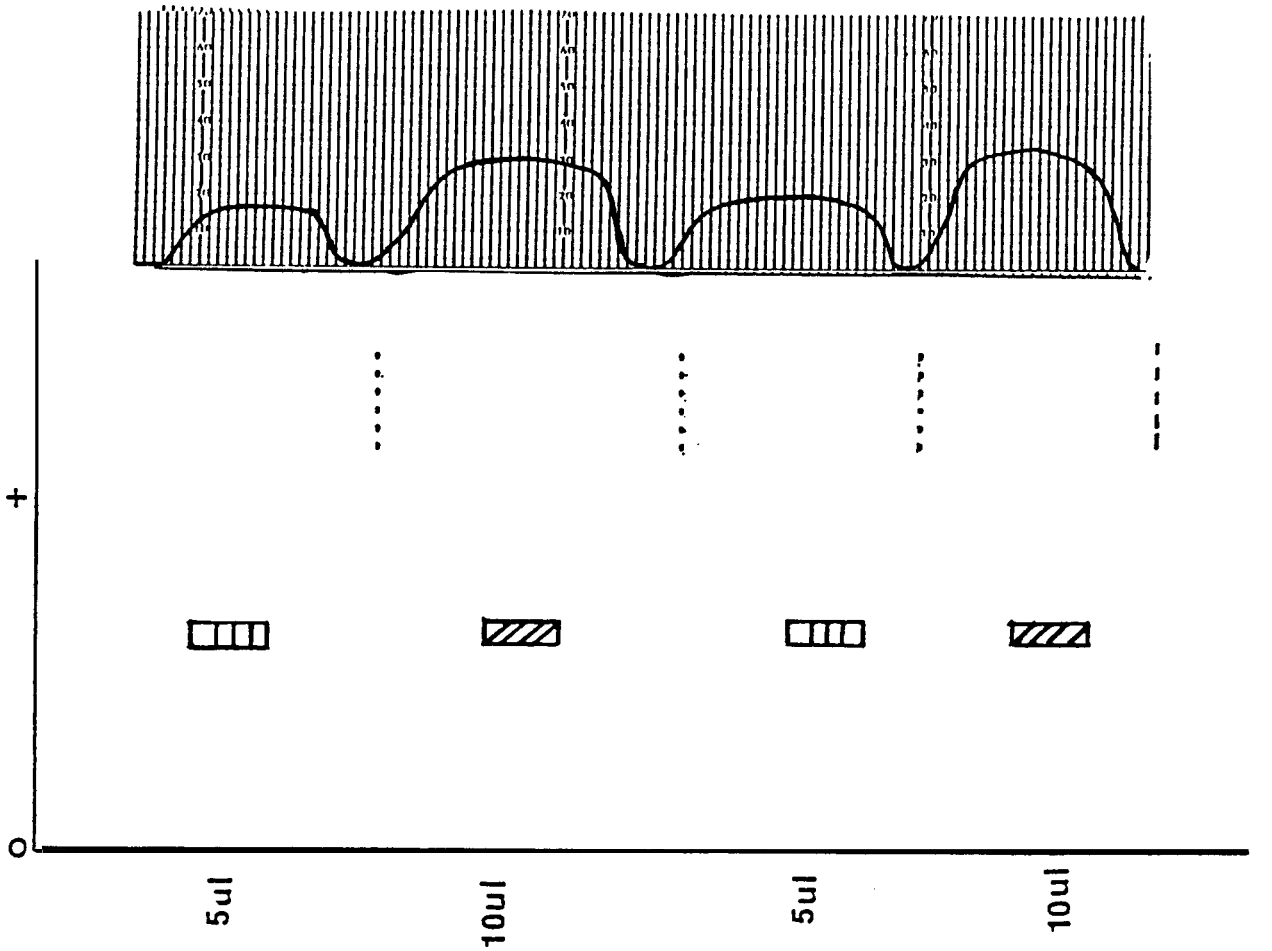
Fig. 15B represents the plasmodia. Larger bands are seen in most cases, with crosses Pan 2-4 wt 1-12 X Ph1A^{2}, PhlA^{2} X Pan 2-441^{8} and Pan 2-441^{8} X CR5-5A^{2} 1-12 being the least active and CR5-5A^{2} X PhlA^{2} and Pan 2-4 wt 1-12 X CR5-5A^{2} 1-12 being the most. Pan 2-4 wt 1-12 X Pan 2-44A^{8} has the largest band. The silver stain therefore indicates that a considerable amount of protein variability indeed exist in the isolates of D. iridis. This can more than likely be attributed to differences in structural protein than isoenzyme variability.

<- more like
< than to

Figure 12.

Electropherogram Representing Alkaline Phosphatase for Spores.

SPORES



CR5-5A^{2}1-12
X
Ph1A^{2}

Pan2-4wt1-12
X$_{2}$
CR5-5A^{2}1-12

BAND INTENSITY:

= DARK

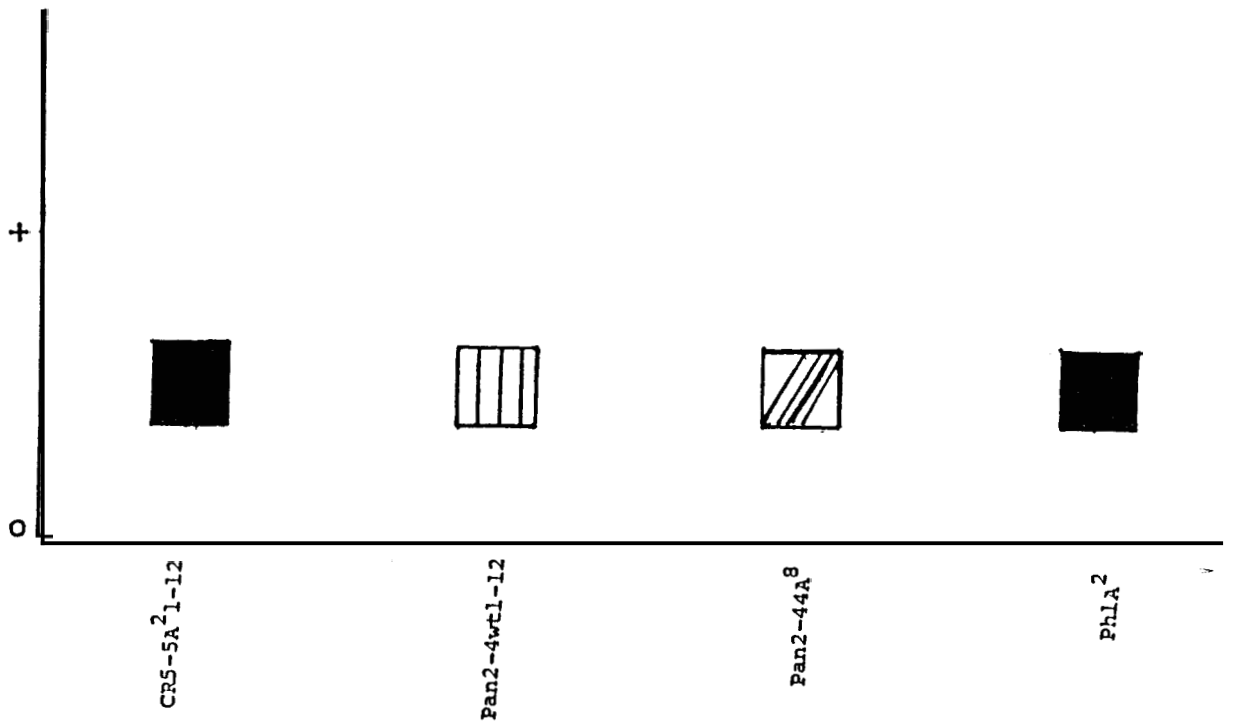
= MEDIUM

= LIGHT

Figure 13.

Electropherogram Representing Esterase D for Myxamoeba.

AMOEBA

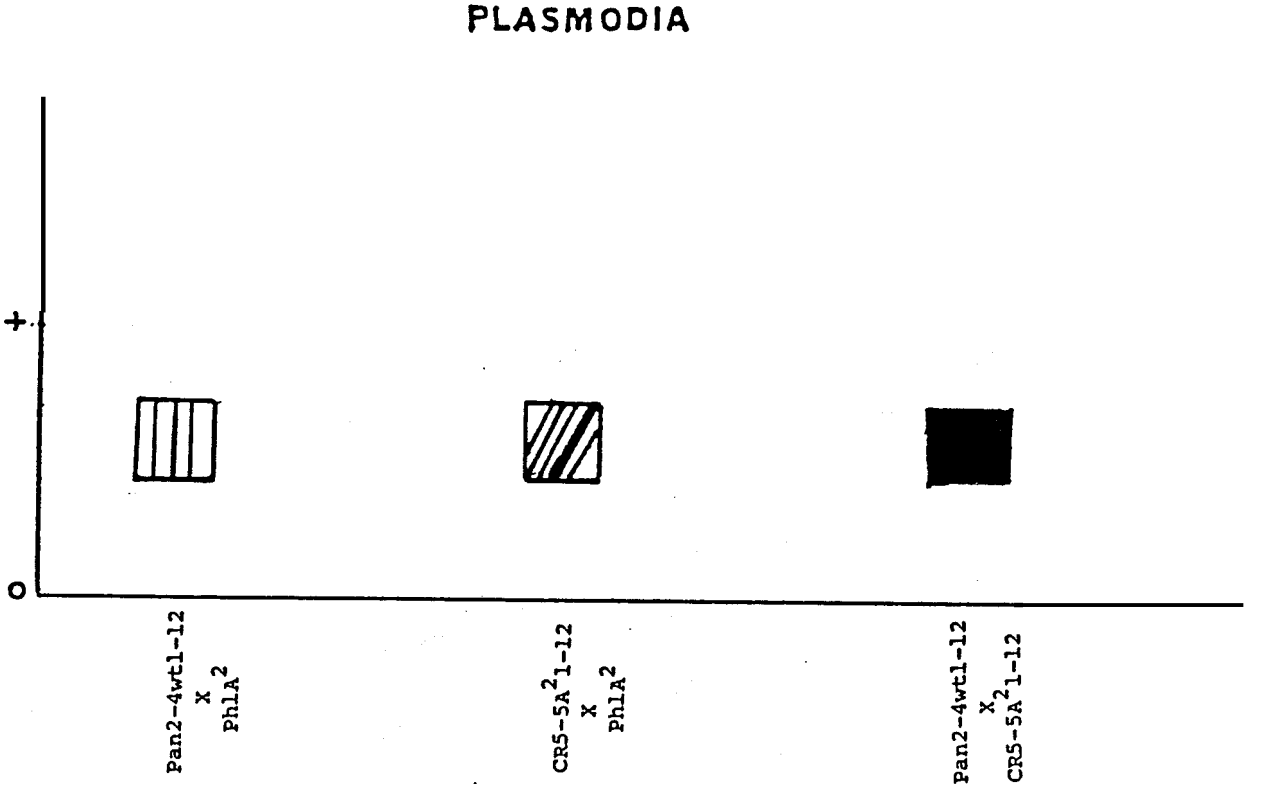


BAND
INTENSITY:

= DARK
= MEDIUM
= LIGHT

Figure 14.

Electropherogram Representing Esterase-D for Plasmodia.


PLASMODIA
+
0
Pan2-4wt1-12 X PhlA2
CR5-5A21-12 X PhlA2
Pan2-4wt1-12 X CR5-5A21-12

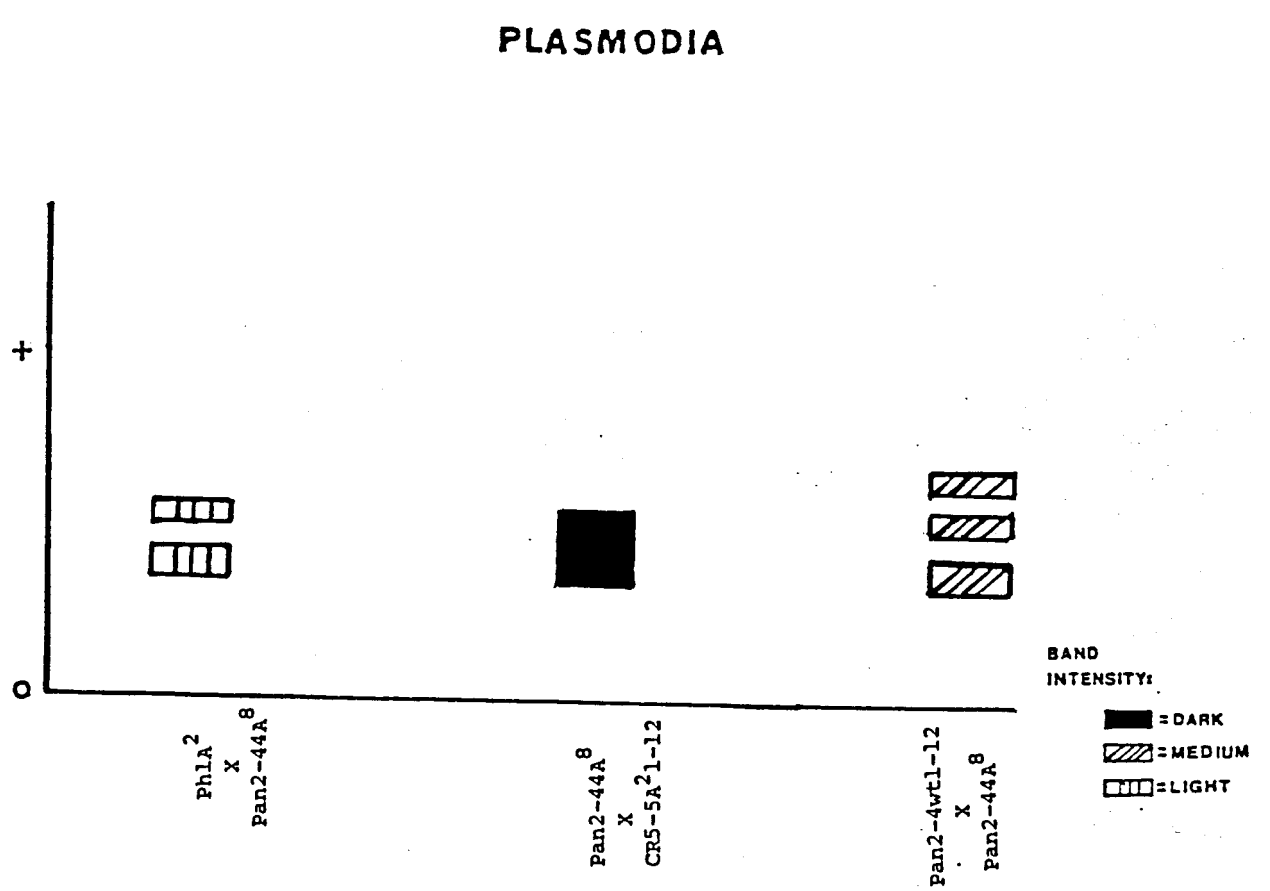
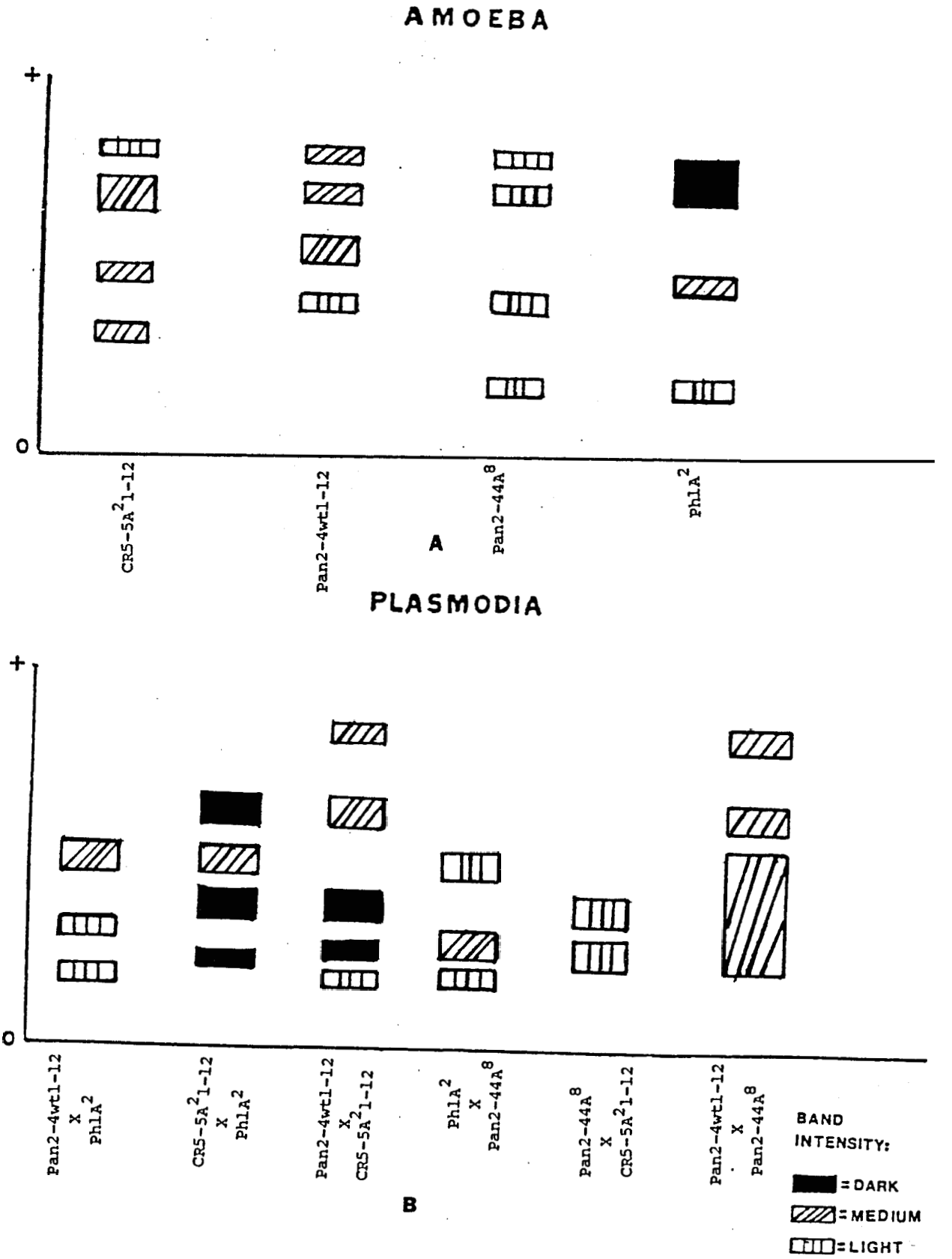

PLASMODIA
+
0
PhlA2 X Pan2-44A8
Pan2-44A8 X CR5-5A21-12
Pan2-4wt1-12 X Pan2-44A8
BAND INTENSITY:
= DARK
= MEDIUM
= LIGHT

Figure 15.

Electropherogram Representing Silver Stain for

(A) Myxamoeba

(B) Plasmodia


AMOEBA
+
0
CR5-5A²1-12
Pan2-4wt1-12
Pan2-44A⁸
PhlA²
A
PLASMODIA
+
0
Pan2-4wt1-12 X PhlA²
CR5-5A²1-12 X PhlA²
Pan2-4wt1-12 X CR5-5A²1-12
PhlA² X Pan2-44A⁸
Pan2-44A⁸ X CR5-5A²1-12
Pan2-4wt1-12 X Pan2-44A⁸
B
BAND INTENSITY:
= DARK
= MEDIUM
= LIGHT

CHAPTER V

Discussion

The Myxomycetes are excellent models for scientific investigation including electrophoretic studies. Some interesting studies already done include the effects of starvation of Physarurn polycephalum as demonstrated by isoenzyme profiles (Hüttermann et al., 1979), and the comparison of isoenzyme profiles between heterothallic and nonheterothallic isolates of D. iridis (Betterley and Collins, 1983).

Since this study involves the investigation of isoenzymes among different geographical isolates within the same species, namely D. iridis, one would expect to find very similar isoenzyme profiles (modes of migration, degree of polymorphism, etc.). If the potential to interbreed among isolates exists, then the isoenzyme profiles will show great similarities. This situation appears to be consistent within groups of Fusarium moniliforme Sheld, as reported by Kathariou (1981). In contrast, noninterbreeding groups that are morphologically indistinguishable exhibited differences in their isoenzyme profiles (Jameson, 1977).

Hence, it was the intent of this study to examine the differences regarding activity and efficiency of certain

metabolic enzymes throughout the life cycle of D. iridis, and to examine the degree of enzyme polymorphism among the designated geographical isolates.

In all cases, different levels of enzyme activities (intensities) were observed utilizing the techniques of electrophoresis, and scanning densitometry. The isoenzyme profiles obtained in this study proved to be quite distinctive and highly reproducible. Quality appeared to be influenced by several factors. Among them are: A) Voltage, electrophoresis was performed at a low, constant voltage (200 V); B) It was not necessary to use detergents in experimental lysates; C) Storage of cell lysates in liquid nitrogen prevented the degradation of the enzymes by proteases within the cell lysate, even after repeated use of the lysate; D) The buffers used for the storage of cell lysates effectively maintained the integrity of the enzymes (Betterley and Collins, 1983); E) Electrophoresis of a concentrated sample of E. coli, the food source of D. iridis, resulted in no isoenzyme patterns and thus no interference; F) The use of sterile oatmeal as a food source for the plasmodia yielded no detectable changes in isoenzyme profiles as previously reported by Franke and Berry (1972). These factors rule out the possibility of extraneous sources introducing variables that may alter the isoenzyme patterns of the specimens under

study.

Isoenzyme Data

Phosphoglucomutase (PGM)

Overall, the activity of this enzyme appears to be greatest in the Myxamoebal stage of the life cycle. Phosphoglucomutase converts glucose-1-phosphate to glucose-6-phosphate which is the first intermediate in glycolysis, as well as the pentose phosphate pathway (Rawn, 1983). The higher activity of this enzyme in the myxamoeba as reflected by activity and the degree of polymorphism seems to indicate that the glycolytic pathway is very prominent this phase of the life cycle of D. iridis. Since these cells achieve logarithmic growth in a short period of time (2-3 days), and thus have the function of increasing cell members in preparation for plasmodia formation, this is expected. It is also important to note that this enzyme plays a prominent role in the hexose monophosphate shunt necessary for the rapid division of cells. Thus PGM couples glycolysis to the pentose phosphate pathway (Rawn, 1983). It should also be noted that this pathway is important in gluconeogenesis necessary for cyst wall formation which also occurs in the myxamoeba when a reduced level of nutrients in the environment is exhibited (Gray and Alexopoulos, 1968).

Isocitrate Dehydrogenase (ICDH)

Isocitrate dehydrogenase converts isocitrate to Succinyl Co-A. This is the first oxidative decarboxylation which leads to the formation of one NADH during one turn of the Kreb's cycle. Even though this enzyme showed relatively lesser activity as opposed to the other enzymes tested, the plasmodia showed significantly greater activity than the myxamoeba, indicating prevalence of the Kreb's cycle in the plasmodia. This correlation is justified by the fact that the plasmodium is a more complex member of the life cycle, and represents the diplophase, the result of the union of the myxamoeba or swarm cells and zygote formation (Yemma and Perry, 1985). Whereas, the myxamoeba are haploid and represent the haplophase of the life cycle. The mentioned-greater complexity corresponds with a more efficient method of energy production in order to compensate for the more diverse metabolic processes evident in the plasmodia including the Kreb's cycle.

Glutamate Oxaloacetate Transaminase (GOT)

Glutamate oxaloacetate transaminase is also an enzyme closely associated with the Kreb's cycle, which functions in the addition or subtraction cleavage of NH_2 groups of amino acids coming in and out of the Kreb's cycle. As with ICDH, the activity of GOT is overall significantly

higher in the plasmodia. This data supports and complements that already presented and discussed previously regarding ICDH activity in the plasodium.

Glucose Phosphate Isomerase (PGI)

Glucose phosphate isomerase is the second intermediate in the glycolytic pathway which functions in converting glucose-6-phosphate to fructose-6-phosphate. This enzyme demonstrated similar intensities in both the myxamoeba and plasmodia for the principal form of the enzyme. However the myxamoeba were more polymorphic for this enzyme exhibiting versatility regarding this enzyme. This data supports the contention that the glycolytic pathway is a dominant mode of ATP synthesis in the myxamoeba. Since this enzyme is prominent in the glycolytic pathway but not part of the pentose phosphate pathway, it would explain the similarity in activity between the myxamoeba and plasmodia, and would also explain the discovery of less activity for this enzyme when compared with PGM in the myxamoeba.

Analysis of the spores indicates a considerable reduction in the activity of the enzymes concerned with growth and energy production for all specimens. Since the spores are concerned with that part of the life cycle characterized by the reduction of metabolic activity and dormancy (Gray and Alexopoulos, 1968), this would be

expected.

Glucose-6-Phosphate Dehydrogenase (G-6-PDH)

Glucose-6-phosphate dehydrogenase is the initial enzyme in the hexose monophosphate shunt, which converts Glucose-6-Phosphate to 6-Phosphoglucono-1,5-lactone. The primary functions of this pathway are A) the synthesis of ribose phosphate, the starting point in biosynthesis of nucleotides and B) the production of NADPH which is necessary for reductive reactions in the biosynthesis of fatty acids (Rawn, 1983). The observed higher activity in the myxamoeba than the plasmodia suggests that this pathway is necessary for the rapid growth rate of the myxamoebal cells and further supports the hypothesis previously discussed regarding the myxamoebal phase of the life cycle in regard to PGM activity. The plasmodia are more complex forms and therefore concerned with assimilation and greater biochemical acitivity than the myxamoeba. This would predicate the importance of pathways which would satisfy greater energy requirements and reduced activity of glycolytic or pentose phosphate pathway enzymes.

It is also worthy to note it has been our observation during the course of this investigation that the plasmodia, once reaching a critical size within a few days, curtails growth but continues rapid assimilation of nutrients.

G-6-PDH showed little or no activity in the mature spores, demonstrating the inactivity of this pathway in this particular stage as explained previously.

Alkaline Phosphatase

Even though it is has been possible to demonstrate the activity of this enzyme in animal tissues, there is considerable confusion concerning its activity in lower organisms (Gahan, 1984). This enzyme, however, is known to cleave phosphate groups from a variety of substrates which may be important in the synthesis of ATP. Alkaline phosphatase activity was somewhat higher in the plasmodia, where metabolism is somewhat higher and therefore energy production. As expected, the activity in the spores was greatly reduced.

Esterase D

Esterases represent a broad spectrum of enzymes in plants and animals. They function primarily in the hydrolysis of carboxylic acid esters of alcohols, phenols and naphtols resulting in phosphate groups necessary for DNA synthesis, ATP synthesis and regulatory factors concerning enzymes. The activity of this enzyme was virtually the same in both the myxamoeba and plasmodia with the plasmodia showing some polymorphism for this enzyme. Once again this supports the contention that this is due to the greater

complexity of the plasmodium.

In conclusion this study demonstrates that the conversion from one phase of the life cycle or morphological form in D. iridis is accompanied by a change in complexity and therefore differential activity of certain metabolic pathways and enzymes and therefore chemical requirements. In addition, the isolates used in this study although demonstrating in some cases different levels of enzymatic activity among themselves also demonstrated great similarity in isoenzyme profiles and thus supports the designation of a single genus and species for these organisms. The silver stain procedure for total protein indicates there is considerable variation however in total protein when interbreeding cones are examined. This can be attributed to structural protein in view of the similarities found in enzyme patterns. However, it appeared that some isolates were more efficient than others for a given enzyme as are different morphological forms within the life cycle thus supporting the fact that the designation of varieties within the species is justified.

The changes in metabolism when going from one stage of the life cycle to the next can be summarized in the following way. The glycolytic enzymes (PGM and PGI) which showed the greatest metabolic activity in the myxamoeba,

demonstrated effectively that the myxamoeba are able to metabolize glucose and its derivatives efficiently. Thus glycolysis is the primary mode of glucose metabolism. The high activity of PGM and G-6-PDH indicate that the pentose phosphate pathway is also prominent due to the rapid growth exhibited by these cells, and because gluconeogenesis has the potential of occurring, resulting in the synthesis of glucose and other carbohydrates which allows for encystment of these cells when nutrients are depleted. Since plasmodia are essentially without cell walls and diploid, thus more complex forms (Alexopoulos, 1968), in comparison to other morphological forms within the life cycle of D. iridis, the need for gluconeogenesis would be less evident. It exhibits limited potential growth and a high rate of nutrient assimilation.

The primary mode of carbohydrate metabolism in the plasmodia was found to be by way of the Kreb's cycle. ICDH and GOT demonstrated considerably higher activity in the plasmodia, indicating that a high rate of assimilation of nutrients and energy production utilization occurs in this stage of the life cycle. Thus the need for a more efficient mode of metabolism arises.

Mature spores demonstrated decreased levels of enzyme activity, as expected, since they are not

metabolically active (Gray and Alexopoulos, 1968). G-6-PDH demonstrated no activity which would indicate a lack of DNA synthesis in this stage. Likewise, the enzymes PGM, ICDH, GOT and Esterase-D exhibited no metabolic activity in the sporulation stage. Finally, even though D. iridis is a relatively primitive organism, it appears to have a rather complex cell cycle as determined by isoenzyme analysis performed in this study. It continues to be an important research tool that can further enhance our understanding of metabolism and cellular differentiation by answering many perplexing biochemical problems, which up to this point may not be fully understood.

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