A STUDY OF THE VARIOUS DIFFERENTIATION PATTERNS OF THE MYXOMYCETE PHYSARUM FLAVICOMUM

A Thesis

Presented to

the Faculty of the Department of Biology

University of Houston

In partial fulfillment of

the Requirements for the Degree

Master of Science

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by

John Lloyd Brice

December 1971

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ABSTRACT

Physarum flavicomum variety 1 was cultured in a semi-defined medium developed by Henney and Henney. Hematin and yeast extract were essential for growth of this organism in the semi-defined medium. The organism was harvested at log phase growth and under proper conditions allowed to differentiate into a sclerotial body and a fruiting body.

The conditions necessary for differentiation of the organism into its various morphogenetic forms are quite diverse. The sclerotial phase of the organism requires an adequate starvation period following its harvest, coupled with the absence of light. The fruiting body, or spore stage, requires a similar starvation period after its harvest, however a light source is essential if the organism is to complete its differentiation cycle. As well, conditions favoring the formation of the fruiting body stage are much more critical for the sporangium than for the sclerotium.

Enzyme assay experiments were performed on the plasmodium as well as the differentiating sclerotium of the organism. The data obtained reveals a definite fluctuation in the activities of LDH, MDH, and G-6-PO_4 over time. Disc-gel electrophoresis was performed on the plasmodium and differentiating sclerotium of the organism and found to be positive for the reactions studied. The enzymes tested were LDH, MDH and G-6-PO_4. The LDH and G-6-PO_4 gave rise to a single band pattern,

while the MDH revealed two bands, the mitochondrial form of the enzyme preceding the cytoplasmic.

Amino acid analyses on the plasmodium, the spores and the differentiating sclerotium reveals definite morphogenetic alterations in the pool of available amino acid content of the organism against time.

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INTRODUCTION

Tremendous progress has been made in all areas of biology during recent years, and the rather specialized area of mycology which deals with the study of the Myxomycetes has been of no exception. The number of workers in this field has greatly increased due to the fact that the Myxomycetes provide an excellent means of attacking a wide array of biological problems. Myxomycetes, or true slime molds, are being used more and more each day in probing out certain biochemical reactions which precede or accompany many physiological and morphological events found to occur in other living systems (1).

Much attention has been focused on the Myxomycetes in recent years because of their diverse life cycle.

The initial stage of the life cycle of the Myxomycetes consists of a somatic phase referred to as the plasmodium. This free-living, acellular, multinucleate, mobile assimilative phase is converted into one or more fruiting bodies which in turn produce spores. The spores, under proper conditions, will germinate and give rise to swarm cells or to non-flagellated myxamoebae, which in turn forms zygote that yields the plasmodium. However, there is still another structure that may play an important role in the survival of the Myxomycetes even though its formation is not necessary for a completion of the life cycle. This is

a highly resistant structure which may be formed directly from the plasmodium when it is subjected to adverse environmental conditions; it represents a resting or dormant phase of the plasmodium and is referred to as the sclerotium (1).

Due to the discovery of the minimal organic requirements for growth of a myxomycete plasmodium by Daniel and Rusch (2) in 1963, laboratory cultivation of the Myxomycetes have become somewhat easier to achieve. Since the medium would not support the growth of other myxomycete, subsequent researchers have developed their own growth media in order to cultivate pure cultures of other species.

Henney and Henney (3) in 1968 report good growth of the plasmodiums of Physarum rigidum and P. flavicomum variety 1 and variety 2 in axenic cultures. Their medium would also support the growth of Physarum polycephalum as well. Later, Henney and Lynch (4) determined the minimal organic requirements for the growth of P. flavicomum, P. polycephalum and P. rigidum. The semi-defined medium used for cultivation of the organisms consisted of yeast extract, casein hydrolysate, dextrose, salts, trace elements, and hematin.

Daniel and Rusch (5) report good plasmodial growth of P. polycephalum in pure culture at pH 5.0 when agitated at 170 reciprocations per minute in the dark for 66 hours. The result is a log phase culture of tiny, individual microplasmodia which quickly coalesce into

a one unit structure if left to settle out in the flask. These microplasmodia are quite distinct from the large surface-grown plasmodia characteristic of the Myxomycetes.

The process of sporulation in the Myxomycetes is a very striking phenomenon. Sporulation, or fructification, entails the conversion of the assimilative structure, the plasmodium, into one or more fruiting bodies which house the spores of the Myxomycetes. Only rarely does one find the somatic and reproductive phases existing simultaneously in the same individual. The process of sporulation in the Myxomycetes is one of complete committal as it is impossible to reverse the process once it has begun. At present there is no known way of predictably maintaining the plasmodium as an active assimilative body once it has been committed to sporulate (1).

It is not clearly understood why the myxomycete plasmodium suddenly curtails all its assimilative processes and initiates its reproductive phase, although a variety of environmental factors have been definitely linked to the fruiting process by several investigators.

Miller (6) observed the sporangia of P. cinereum and found for the most part that it developed at night. Similar observations have been afforded by other researchers such as Jahn (7), Lister (8), and Howard (9). They surmised that the sequence of events giving rise to a morphological change in the plasmodium was made at night.

Goodwin (10) in 1961 reports that the pre-dawn hours are most conducive to development of the sporangia in Comatricha. She observed that fruiting bodies developed readily during the pre-dawn hours, but found if one placed the cultures at 15 C overnight the fruiting process was reduced.

Cayley (11) studied several species of Didymium and concluded from her results that definite rhythmical fruiting exists in the Myxomycetes. She reports that D. difforme possesses a much shorter assimilative phase than either D. nigripes or D. squamulosum. She ended in the conclusion that the factors which cause plasmodia to form fruiting bodies are still unresolved.

Seifriz and Russell (12) conclude from their observations on the fruiting of P. polycephalum that rhythmical fruiting is an innate characteristic of the Myxomycetes.

Camp (13) made the suggestion from his fruiting studies with P. polycephalum that the primary factor responsible for the induction of the sporulation process was depletion of nutrient materials. He found that the length of the vegetative phase in P. polycephalum was very much shortened by the withholding of a nutrient supply.

Gray (14) expressed the opinion that such fruiting rhythms might well exist in the Myxomycetes; however, he set out to challenge the innate inalterability of the fruiting rhythms through environmental

manipulations. Gray's consensus was that it should be possible through proper environmental manipulations to alter the assimilative phase of the plasmodium. He found that light was required to initiate fruiting in the yellow-pigmented plasmodial forms, but that the non-pigmented forms such as P. nutans sporulate equally as well in the presence or absence of light. Gray found as well that under conditions of continuous illumination the time required for the organism to produce fruiting bodies varied inversely with a function of light intensity. He also found that if starved cultures were exposed to a continuous illumination system the identical relationship between time and light intensity prevailed; however, the time required for sporulation was drastically reduced. In general, Gray (14) concluded that fed cultures required more time to sporulate than did starved cultures. He also concluded that as lamp size increased, the time required for both starved and non-starved cultures to sporulate was markedly shortened.

Von Stosch (15) studied the relationship of light intensity to fruiting in D. nigripes and found that there was a certain optimum intensity for maximum sporulation in this organism. Further increases to either side of this maximum intensity led to decreased percentages of fruiting. Unlike Gray, von Stosch employed a wide range of intensities in his experimentation. His maximum value for 100 percent fruiting was 600 Lux units.

In a follow-up paper using a tungsten lamp as his light source, Gray (16) found that shorter wavelengths of visible light were more conducive to the fruiting of P. polycephalum. At a temperature of 25 C and pH 3, Gray used starved cultures of P. polycephalum and subjected them to various wavelengths of light. He found that 90% of his cultures fruited at a wavelength of 4360 Å and 224 erg/cm^2/sec while fruiting dropped to 24% for wavelengths of 5790 Å and 392 erg/cm^2/sec. Intermediate between these wavelength ranges, Gray found P. polycephalum to fruit 62.3% of the time at 5460 Å and 224 erg/cm^2/sec.

In contrast to Gray's findings, Rakoczy (17) conducted monochromatic light studies on the myxomycete P. nudum and found 330 nm to be the most effective wavelength for inducing sporulation in this organism.

Straub (18) found that D. nigripes would not sporulate in the dark or in green light but would fruit in white, blue or red. As well, Straub's findings on the length of the illumination period and light intensity coincided with Gray's earlier conclusions confirming an inverse relationship between these two parameters. Straub further found that the length of the period of illumination required for sporulation could be shortened by feeding plasmodia on previously illuminated plasmodia. This finding suggests the existence of a substance or substances synthesized photochemically which could be instrumental in triggering the

onset of the sporulation phase in the organism.

Gray (16) prepared a crude acetone extract of the myxomycete P. polycephalum in order to determine the absorption spectrum of the plasmodial pigment. Gray's interest was focused on the possibility of a relation between pigmentation and light requirement for sporulation. This would set up the possibility that plasmodial pigments in the Myxomycetes function as photoreceptors. From his data, Gray achieved maximum absorption values for the acetone extract at a wavelength of 4300 Å. This value is concordant with his earlier findings concerning maximum fruiting percentages at a wavelength of 4360 Å. Gray's conclusion was that the action spectrum for sporulation agrees most favorably with the absorption spectrum of the plasmosial pigment. It would seem feasible therefore, to assume that the yellow pigment in P. polycephalum is a photoreceptor, an hypothesis supported by Daniel and Rusch (5).

Carotenoids (19, 20, 21), flavoproteins (21), and pteridines (21) have all been suggested as possible photoreceptors for light-induced sporulation of fungi.

Leach (22) has found that fungi grown on malt extract agar and then exposed to monochromatic radiation from a high-intensity quartz Mercury-arc lamp will fruit at wavelengths between 238 nm and 336 nm. Wavelengths of 238 nm and 313 nm were the most effective in initiating

sporulation; whereas, wavelengths of 405 nm and 546 nm did not induce fruiting even at very high dosages. The dose (intensity x time) necessary to induce sporulation decreased with shorter wavelengths. The higher the intensity, the shorter the minimum exposure necessary to induce phcnidial formation.

In addition to light, a variety of other environmental factors have been associated with the fruiting process.

D. squamulosum was found to be maintained in an active state for an indefinite length of time if frequent transfer to fresh nutrient material was performed. However, sporangia were soon formed if a portion was transferred to a moist, nutrient-free substrate. Thus, by removing the nutrient supply, the sporulation process was initiated (23).

Camp (24) cultured P. polycephalum on wet filter paper in a moist chamber using rolled oats as a source of nutrients and reported that, with other conditions being favorable, plasmodia would live and grow as long as nutrient supply was provided; however, if the oats were not changed after depletion and replaced, fruiting ensued.

Spores of the organism Mucilago crustacae were germinated in water by Schure (25) who found that the young plasmodia which formed would never sporulate on an agar medium consisting of bakers yeast. Yet, when they were allowed to spread on moist, porous flower pots, sporulation was immediately obtained.

Acidity of substrate is a very important parameter in sporulation of the Myxomycetes as evidenced by Gray (26) in his work with P. polycephalum. Gray found that high acidity (pH 3.0) was most favorable for fruiting of P. polycephalum. However, he found an interrelationship between pH and temperature as well. In the temperature range of 21 C to 32.5 C, the lower the temperature, the wider the pH range over which the organism could sporulate.

Collins (27) found a similar relationship between pH and temperature in his studies with Fuligo cinerea. He found that both temperature and pH affected fruiting. He found no fruiting at 30 C regardless of the pH of the substrate.

A precise temperature limit for sporulation has not been resolved for any of the Myxomycetes. However, from the data of Gray (26), one would place the upper temperature limit at 32.5 C for the organism P. polycephalum and 30 C for F. cinerea, based on Collin's (25) findings. A lower temperature limit for sporulation in the Myxomycetes has not been firmly established.

Scholes (28) noted that the age of the culture was the most important factor influencing sporulation in F. septica. Various nutrients, type of substratum, light, temperature, starvation, or dessication, failed to induce sporulation. There was a significant difference, however, in the percentage of sporulating cultures between two groups,

one subcultured every four weeks, the other not subcultured until sporulation occurred. In the former, 4% of the cultures sporulated within 28 days; in the latter, 48% of the cultures were positive for fruiting.

In a recent publication of Mohberg and Rusch (29), it was assumed that the spores of the subline of P. polycephalum had lost their ability to undergo meiosis due to prolonged culture in the laboratory. The spores were only 48 hours old and produced no amoeba colonies when plated with E. coli on agar. Experiments were therefore done with plasmodia, spores and spherules of several sublines which had been in culture for a shorter time and were found to still produce viable spores.

Daniel and Rusch (30) have found that glucose added to culture plates of P. polycephalum four days prior to illumination, three days after illumination, and at intermediate times was a strong inhibitor of sporulation. However, Daniel (31) states that glucose inhibits sporulation before but not after illumination, and interprets this in terms of a light-induced alteration of cell permeability, especially to glucose.

In another paper by Daniel and Rusch (32), it was found that niacin analogs had an inhibitory effect on sporulation if added at the beginning of dark incubation but did not inhibit fruiting if added at the end.

Still and Ward (33) found indole-3 acetic acid (IAA) in spores of P. polycephalum but were unable to detect any trace of the compound in the plasmodia or the intermediate stages during morphogenesis. They found that the absence of the compound in the plasmodium and its intermediate stages were due to its enzymatic destruction. Challenging the enzyme with catechol, chiorogenic acid, caffeic acid, epinephrin, and noradrenalin, no inhibitory effects were found; thus, the enzyme differs from the classical IAA oxidase. As well, the activity of the enzyme was not enhanced by manganous ion or 2-4-dichlorophenol as is the classical IAA oxidase.

Still (34) freed homogenates of dark grown plasmodia from pigment, and then placed aliquots in light and dark for one hour. The light exposed homogenate could not enzymatically utilize IAA, but that kept in the dark could. Homogenates still containing the pigment did not demonstrate this dark reversal. Therefore, it would seem that light has an inhibitory effect upon the IAA binding system in the plasmodium of P. polycephalum.

Daniel and Rusch have contributed much research in the area of elucidating the factors responsible for the initiation of the sporulation process (30, 31, 35). These workers cultured plasmodia of P. polycephalum in liquid, shake, bacterium-free culture on tryptone-yeast extract-glucose medium for three days, after which time the

small, discrete plasmodia that had developed was harvested and allowed to fuse (coalesce) into a single plasmodium. This in turn was incubated for four days at 21.5 C in the dark on a $CaCo_3$-buffered salts medium which contained 0.01% niacin and 0.01% niacinamide. After four days of incubation in the dark, the cultures were exposed to light for 2 hours and then returned to darkness. Sporulation was completed in 12 to 16 hours following the light exposure. According to Daniel and Rusch, the obligatory conditions for fruiting of P. polycephalum are: (a) an optimal growth age occurring just prior to maximal growth and at a time when the medium nutrients are exhaustated, (b) four days of incubation on a medium containing only inorganic salts and niacin or tryptophane (both DPN and TPN can also serve as niacin sources), and (c) subsequent illumination with light of wavelengths between 350 mu and 500 mu. This procedure seems to be most effective in initiating the complete sporulation process in the myxomycete P. polycephalum.

Jump (36) describes the sclerotization of P. polycephalum as an orderly process in which the following sequence of events occurs: (a) cessation of protoplasmic streaming, (b) gelation of the whole structure, (c) distribution of nuclei, (d) depositing of wall, (e) completion of macrocyst formation, (f) hardening of the sclerotium, and (g) shrinkage of nuclei to about one-half their original diameter.

The conditions which bring about sclerotization of P. polycephalum in the laboratory are best described by Goodman, Sauer, Sauer and Rusch (37). These workers achieved spherulation in about 30 hours by innoculating 2 ml of a log phase plasmodial suspension (obtained from a 66 hour shake flask) into a salts medium specially prepared to induce spherulation.

Lonert (38) developed sclerotium from the myxomycete P. polycephalum by overfeeding the organism with rolled oats and then drying it in a hot-air oven. Storage under refrigeration produced no change in vitality of the sclerotium, but storage at room temperature or prolonged dessication increased the length of time required for transformation of sclerotia to plasmodia from 2.5 hours to 7 and 17 hours, respectively. The sclerotia prepared in this manner were viable after 12 to 16 months.

Zeldin and Ward (39, 40) studied the protein profiles of the slime mold P. polycephalum both before and after morphogenesis, using acrylamide gel electrophoresis as their index of measure. They found several changes in the protein content of the plasmodia before there were any visible evidences of differentiation. In particular, the specific protein, α -amylase, which mobilizes nutrient polysaccharide, was found to decrease 60% in activity after differentiation.

Huttermann, Porter, and Rusch (41) studied the activities of

seven enzymes during the first 24 hours of spherule formation in cultures of P. polycephalum. These enzymes were: isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, acid phosphatase, phosphodiesterase, B-glucosidase, and histidase. Isocitrate dehydrogenase and glucose-6-phosphate dehydrogenase showed a decrease, glutamate dehydrogenase and phosphodiesterase increased about eight-fold, acid phosphatase about two-fold, B-glucosidase showed an activity peak at about 15 hours after starvation which decreased after 24 hours to its original value, and histidase showed no significant activity change during the period.

Glutamate dehydrogenase was studied more closely in a later paper by Huttermann, Elsevier, and Eschrich (42). These researchers set out to discern whether the increase in activity during differentiation of P. polycephalum was due to enzyme activation or to de novo synthesis. They concluded from density shift studies that the de novo synthesis did play an important role in the observed increase in activity of the enzyme during spherulation.

Tatum (43) describes a morphological mutant in Neurospora crassa known as a colonial mutant. Mutations at a specific gene locus appear to alter the structure of the glucose-6-phosphate dehydrogenase, leading to an intracellular accumulation of glucose-6-phosphate, and ultimately to a striking change in the growth pattern

of N. crassa.

Spores have become attractive to biochemises as an object for the study of cellular differentiation and morphogenesis.

Kornberg (44) attempted to compare the extent of protein turnover in the mother cells of Bacillus subtilis with that in its forespore by isolating a homogenous population of sporulating cells. He found that protein breakdown and resynthesis during sporulation amounted to 67% and 52%, respectively.

Monro (45) measured the decrease of radioactive protein in B. thurigiensis sporulating in a medium containing unlabeled amino acids, and observed a loss of 10% per hour while total protein remained nearly constant. Monro concluded, however, that isotopic labeling failed to reveal protein turnover under certain conditions, presumably because some amino acids fail to equilibrate with the internal pool.

Nelson and Kornberg (46) report that pools of free amino acids in spores and vegetative cells of B. megaterium were much in contrast with one another. Spores of the organism were rather restricted in free amino acid content while the vegetative cells contained a wide variety. L-glutamic acid was the predominant component of the amino acid pools of vegetative cells and of spores of several species of Bacillus. Free L-lysine and L-arginine were found in spores of

some species. Spore extracts also contained several ninhydrin-reactive compounds which could not be identified with commonly occurring amino acids.

Kornberg (44) found that in contrast to L-glutamate, the levels of a number of other amino acids are vanishingly low. For instance, Kornberg suggests that arginine and lysine are not invariably found in spores. In all cases studied, the amino acid pool of dormant spores proved to be small.

The purpose of this research was to establish a reliable fruiting system for the myxomycete P. flavicomum variety 1, as well as to test a variety of parameters as to their facilitative or diminutive effects on sporulation. Enzyme assays coupled with gel-electrophoresis were also performed on the plasmodia and several phases of the sclerotium of P. flavicomum in order to observe any changes in the activities of lactate dehydrogenase, malate dehydrogenase, and glucose-6-phosphate dehydrogenase as the organism progresses through its morphogenetic sequences. Protein content of these various morphogenetic stages were closely followed throughout the differentiation process. In addition, amino acid analyses were performed on the soluble pools of the plasmodial stage, the early sclerotial phase, the completed sclerotial phase and the spores, in order to observe any changes in the amino acid pattern

of the vegetative plasmodial phase over the differentiated states of the organism.

MATERIALS AND METHODS

Organism. P. flavicomum variety 1 was the primary organism of research in this study, although P. polycephalum was used as a control in some selected experiments; however, both organisms are cultured in precisely the same fashion on a semi-defined media developed by Henney and Henney (3).

Media. The semi-defined media used for culturing purposes in this research is listed in Table 1. The basal salts solution was made up in 10X concentration at a final pH of 4.0. All pH values were registered on a Beckman Zeromatic pH meter (Beckman Instruments Inc., Fullerton, California). Each component of the basal salts and trace elements was added successively in the order listed to assure solubility. Both trace elements and basal salts were kept frozen and thawed just before use.

Hematin solution was prepared by dissolving 0.25g of heme (Sigma Chemical Co., St. Louis, Mo.) in 100 ml of 1% NaOH. The final concentration was 0.25%. This solution was autoclaved separately and added to the sterilized semi-defined media after it had cooled to room temperature (0.1 ml of heme per 100 ml of media). The hematin was kept frozen and thawed before use.

TABLE 1

Medium for Growth of Physarum Flavicomum Variety 1

Basal Salts Solution (pH 4.0)		
Component		g/liter
Citric Acid	(Fisher Scientific Co., Fairlawn, N.J.)	29.78
K_2HPO_4	(J. T. Baker Co., Phillipsburg, N.J.)	33.11
NaCl	(J. T. Baker Co., Phillipsburg, N.J.)	2.50
$MgSO_4 \cdot 7H_2O$	(J. T. Baker Co., Phillipsburg, N.J.)	1.00
$CaCl_2 \cdot 2H_2$	(J. T. Baker Co., Phillipsburg, N.J.)	0.50
glass distilled H_2O	..... to	1000 ml

Trace Element Solution		
Component		g/95 ml
Citric Acid	(Fisher Scientific Co., Fairlawn, N.J.)	5.0
$ZnSO_4 \cdot 7H_2O$	(J. T. Baker Co., Phillipsburg, N.J.)	5.0
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	(J. T. Baker Co., Phillipsburg, N.J.)	1.0
$CuSO_4 \cdot 5H_2O$	(J. T. Baker Co., Phillipsburg, N.J.)	0.25
$MnSO_4 \cdot H_2O$	(J. T. Baker Co., Phillipsburg, N.J.)	0.05

TABLE 1 (cont'd)

Component		g/95 ml
H_3BO_3 • Anhydrous	(Fisher Scientific Co., Fairlawn, N.J.)	0.05
$Na_2MoO_4 \cdot 2H_2O$	(Fisher Scientific Co., Fairlawn, N.J.)	0.05
$Co(Cl)_2 \cdot 6H_2O$	(J. T. Baker Co., Phillipsburg, N.J.)	0.05

Dissolve successively in 95 ml of glass distilled H_2O at room temperature.

Complete Media

Component		g/liter
Basal salts (pH 4.0)		100.0 ml
Trace elements		0.1 ml
Dextrose	(J. T. Baker Co., Phillipsburg, N.J.)	5.0
Yeast Extract	(Difco Lab., Detroit, Mich.)	5.0
N-Z Casein Hydrolysate	(Sheffield Chem., Oneonta, N.Y.)	5.0
glass distilled H_2O	..... to	1000 ml

Methods of Culture. P. flavicomum variety 1 was grown in the dark at 22 C in 500 ml Erlenmeyer flasks containing 200 ml of semi-defined media on a rotary shaker (New Brunswick Scientific Co., New Brunswick, New Jersey) at 170 reciprocations per minute. Prior to innoculation of the media, the flasks were plugged with metal caps and/or cotton stoppers and autoclaved at 15 psi for 15 minutes.

All autoclaved flasks were incubated for three days at room temperature before inoculation in order to assure no chance of contamination. The plasmodia used for inoculation was grown for 66 to 72 hours on the semi-defined media.

Log phase growth of the organism was achieved in 66 hours after inoculation with 2 ml of packed microplasmodia.

Method of achieving sporulation. The fruiting method devised by Daniel and Rusch (30) was used in order to achieve sporulation in the organism P. flavicomum, with, however, slight modifications. Log phase flasks were tilted to a 45^{o} angle in order to allow sedimentation of the microplasmodia. This process should not be carried out any longer than 5 minutes as the organism is not being aerated. After the organism has sufficiently settled to t he bottom of the flask, the spent media can be aseptically decanted off, leaving the microplasmodia behind. At this point the flask is reflamed and the cotton plug replaced, leaving only the organism and trace amounts of the

semi-defined media behind. The microplasmodia is then washed once with a solution of basal salts and distilled water (30) at pH 4.0. The washing can take place in the original log phase flask (still housing the organism) by the aseptic addition of 125 ml of the salts solution. The flask is rotated gently by hand to wash down any of the microplasmodia adhering to the sides of the glass as well as to assure the complete re-suspension of the organism in the salts solution. The microplasmodia is allowed to settle again and the salts solution is now ready for a final decantation. Care should be taken in aseptically pouring off the salts solution from the washed cell suspension as the myxomycete is extremely sensitive to heat shock (26). After flaming the flask (once or twice) slowly decant the 125 ml of salts solution in to a waste receptacle so as to cool the neck of the flask before the cells begin to slide down. As the salts media is gradually removed and the washed cells begin to emerge, a 150 mm Kimax petri-dish (W. H. Curtain Co., Houston, Texas) containing Whatman No. 1 filter paper cut to a diameter of 140 mm, supported by 5 mm solid Kimax glass beads (W. H. Curtain Co., Houston, Texas) should be previously sterilized and ready to catch the microplasmodia as it is decanted out. The petri-dish and its contents should be autoclaved at 15 psi for 15 minutes and vacuumed dry for 30 minutes inside the autoclave. After vacuuming, the petri-dishes should

be dessicated in a hot-air oven overnight at 70 C. The petri-dishes with glass beads and filter paper must be allowed to equilibrate to room temperature 15 minutes before the decantation process takes place. With the microplasmodia now at the lip of the Erlenmeyer flask, gently raise the lid of the petri-dish and quickly decant the organism onto the center of the filter paper in one quick motion. All such procedures should be carried out in a sterile environment, such as a transfer, room sprayed with a 200 ppm Roccal solution (Winthrop Laboratories, New York, N.Y.) and/or illuminated with ultra-violet lamps prior to handling the organism.

Immediately following the decantation process transfer the petri-plates with the microplasmodia to a 22.5 C incubator (Precision Scientific Co., Chicago, Illinois). Allow the microplasmodia to fuse (coalesce) into a one unit structure. The time required for coalescence is 4 to 5 hours (30). After 4 to 5 hours have elapsed return the petri-plates to the transfer room and add 25 ml of sterile sporulation media, consisting of a double-distilled water adjusted with 1 N HCl to a pH of 4.0. The addition of the sporulation media is facilitated by the use of a 25 ml Pyrex pipette (W. H. Curtain Co., Houston, Texas), autoclaved at 15 psi for 15 minutes prior to the experiment. The organisms are now ready to begin their starvation period. The petri-plates with the coalesced microplasmodia and sporulation media

are returned to the 22.5 C incubator, in the dark, and allowed to incubate for 96 hours. During this dark-period, the microplasmodia will crawl around the inside of the petri-plate and eventually assume the form of the plasmodial state, characteristic of the Myxomycetes.

Following 96 hours of incubation, the plasmodia are now ready to be exposed to light. The illumination system used consists of four 100 watt Ken-Rad incandescent light bulbs (Ken-Rad Lamp Co., Owensboro, Kentucky) mounted inside an Environette controlled environmental chamber (Lab-Line Instrument Inc., Melrose Park, Illinois). The lamps are fastened to Afco base plates (Afco Manufacturing Co., St. Louis, Missouri) and suspended from the bottom surface of the upper shelf inside the Environette chamber. The naked lamp bulbs will then be facing downward, 10 inches above the surface of the next shelf. The maximum output per 100 watt lamp is 1640 lumens. This would be analagous to 9.84×10^7 ergs/sec or, at a uniform distance of 1 foot from the lamp source, 6560 foot-candles. The temperature was held constant at 23 C throughout the light exposure. The time of illumination varied from 2 hours to 24 hours, depending on the experiment at hand.

After illumination the petri-plates were either allowed to remain in the Environette and undergo a dark-period, or they were carefully removed and placed back into the 22.5 incubator for the duration

of their dark-period. Sporulation was completed in 20 hours after placement in the dark incubator, which followed the end of the illumination period.

A variety of sporulation media were tried in order to test their facilitative or diminutive effects on fruiting. Salts-niacin solution was prepared in a concentration of 0.098 g/l. The solution contained 0.098 g of niacinamide and 0.098 g of nicotinic acid (Sigma Chemical Co., St. Louis, Missouri) in addition to 100 ml of basal salts (10X), brought to 1 liter. Two liters were prepared. One liter was filter sterilized with a Nalge disposable filter unit, 0.45u grid membrane (Nalge Sybron Corp., Rochester, N.Y.), while the other 1-liter solution was routinely autoclaved at 15 psi for 15 minutes. The solutions were also prepared in the absence of the 10X basal salts in order to test the effect of the free compounds in water. All solutions were adjusted to a pH 4.0 with 1 N HCl or 1 N NaOH.

Sporulation media consisting of various other compounds were prepared: (a) N-Z case (Sheffield Chemical, Oneonta, N.Y.) 0.5 g/l, pH 4.0; (b) adenosine 3' -5' monophosphate (Sigma Chemical Co., St. Louis, Missouri), 0.001 g/l and 0.002 g/l; (c) hematin, 0.25% solution; and (d) catechol, 0.005 M and 0.01 M (Matheson Coleman and Hall Corp., Cincinnatti, Ohio). In all cases, the prepared solutions were brought to 1-liter, or 100%, with glass, double-

distilled water.

A variety of different-colored 100-watt, incandescent flood-lamps were used in order to test their possible effects on fruiting (General Electric Co., Cleveland, Ohio). The colors selected were red, yellow, and blue. Each lamp had a similar lumen output as the Ken-Rad lamp source, the difference between the two systems varying by only 65 total lumens.

Fluorescent lamps (General Electric Co., Cleveland, Ohio) were applied to the illumination system as well, in order to test their effects on fruiting. The intensity output of one 2-foot, 20-watt, cool-white fluorescent bulb is 1300 lumens. The intensity output for a 100-watt, incandescent Ken-Rad bulb is 1640 lumens. Therefore, five 20-watt, cool-white fluorescent buls were used to equilibrate the two systems.

Method of Achieving Spherulation. Log phase flasks of the plasmodium are decanted aseptically (as in the sporulation procedure) into a specially prepared starvation media described in Table 2, in order to achieve spherulation of the organism (37). To avoid problems of insolubility in preparing this media, citric acid was dissolved in 800 ml of double-distilled water and the salts added in the order listed in the Table. The pH was adjusted to 3.8 with 30% KOH, and the final volume was brought to 1 liber. Six 500 ml Erlenmeyer flasks each

TABLE 2

Starvation Media for Achieving Spherulation of Physarum Flavicomum

Complete Starvation Media		
Component		g/l
Citric Acid·H_2O	(Fisher Scientific Co., Fairlawn, N.J.)	4.02
$FeCl_2 \cdot 4H_2O$	(J. T. Baker Chem. Co., Phillipsburg, N.J.)	0.07
$MgSO_4 \cdot 7H_2O$	(J. T. Baker Chem. Co., Phillipsburg, N.J.)	0.60
$CaCl_2 \cdot 2H_2O$	(Matheson, Coleman & Bell, Norwood, Ohio)	1.20
$MnCl_2 \cdot 4H_2O$	(J. T. Baker Chem. Co., Phillipsburg, N.J.)	0.084
$ZnSO_4 \cdot 7H_2O$	(J. T. Baker Chem. Co., Phillipsburg, N.J.)	0.0336
KH_2PO_4	(Matheson, Coleman & Bell, Norwood, Ohio)	0.4

containing 200 ml of semi-defined media plus the 66 hour log phase yield of cells grown from a 2 ml innocula were used to produce the sclerotium. Two of the 500 ml log phase Erlenmeyer flasks were incorporated into one 500 ml flask in order to obtain 400 ml of semi-defined media plus the growth yield of the organism. This procedure can be accomplished through aseptic decantation, following the technique described in the sporulation process. The cells are allowed to settle out at a 45^{o} angle for 5 minutes after which the spent media is aseptically decanted. To the unwashed cells, remaining in the flask, add 200 ml of sterilized starvation media by aseptically decanting it from one flask to the other. The starvation media should be prepared 3 days prior to inoculation, autoclaved at 15 psi for 15 minutes, and allowed to incubate at room temperature before use in the experiment. The remaining two sets of flasks are treated in the following manner: (a) one set is incorporated into the starvation media exactly as was the other, while (b) the remaining set is emersed in 200 ml of salts solution, pH 4.0, and immediately prepared for enzyme assay analysis. There are now three 500 ml Erlenmeyer flasks, two containing microplasmodia and starvation media, and one containing microplasmodia and cold basal salts solution, all of similar volume, $\pm$ 3 ml. The two flasks containing the starvation media are returned to the rotary shaker (170 reciprocations per min) to be removed at a later date.

The remaining flask with the cold basal salts solution is immediately placed in an ice bath. The total volume of cells and media are measured while keeping the solution cold. A 1 ml aliquot is removed and tested for protein by the Lowry method. A 5 ml aliquot is removed and examined for dry weight. In both cases, a 1 and 5 ml pyrex blow-out pipette was used (W. H. Curtain Co., Houston, Texas) and the entire contents to be used in the analysis was blown through completely. The remaining cells and media are then decanted into cold Nalgene 50 ml centrifuge tubes (W. H. Curtain Co., Houston, Texas) and spun at 1500 rev/min (270 x g) for 5 minutes at 4 C in a Servall refrigerated automatic centrifuge (Ivan Servall Inc., Norwalk, Connecticut). The media is decanted off and the cells removed and placed in a cold, porcelain mortar (Coors Serial 522-1, W. H. Curtain Co., Houston, Texas). The cell pellet is just covered with cold (4 C) 0.05 M tris-cl buffer and an equal pellet volume of acid-washed ground glass (Fisher Laboratory Chem. Co., Fairlawn, New Jersey) is sprinkled over the cells. The preparation is ground with a pestle (Coors, Serial 522-1, W. H. Curtain Co., Houston, Texas) in the cold for 10 minutes. Before and after the grinding procedure, the microplasmodia should be checked microscopically to assure complete breakage of the cells. After 10 minutes of grinding the microplasmodia is sufficiently disrupted and the solution is decanted

into cold Corning 15 ml centrifuge tubes (Fisher Scientific Co., No. 8441, Fairlawn, New Jersey). The ground cells are centrifuged at 15,000 rev/min (27,000 x g) for 10 minutes at 4 C. The supernatant is collected and placed in a cold 50 ml beaker and stored on ice. The pellet is resuspended in 1 ml of 0.05 M tris-cl buffer and recentrifuged again. The second supernatant is then added to the first. The crude microplasmodial extract is stored at 4 C to be used for enzyme assay analysis within the hour.

After 24 hours of shaking in the starvation media the second flask is removed for enzyme assay analyses. The cells are entering the initial stages of early sclerotization and shall hereafter be referred to as Phase I sclerota. The procedure for extracting the supernatant is the same as for the microplasmodial stage. A microscopic examination must be performed both before and after grinding with the mortar and pestle, to assure complete breakage of the Phase I sclerotium. After a final resuspension of the pellet and subsequent centrifugation, the supernatant is decanted off and stored at 4 C. Enzyme assay should be performed within the hour.

After 96 hours of shaking in the starvation media the final sclerotial stage, referred to as Phase II sclerotium, is ready to be prepared for enzyme analysis. A similar procedure of extraction is used for the Phase II cells; however with slight modification.

The outer sclerotial wall has hardened considerably in comparison to the plasmodial and Phase I stages. Therefore, 20 minutes of grinding is necessary to completely disrupt the rigid, outer cell wall as well as twice the amount of ground glass per pellet yield. After the final centrifugation, the supernatant is stored at 4 C to await enzyme assay analysis within the hour.

Enzyme Assays. The following assays were performed by methods used and described by other workers: lactate dehydrogenase (LDH) (47); malate dehydrogenase (MDH) (48); glucose-6-phosphate dehydrogenase (Glucose-6-PO_4) (49).

To measure lactate dehydrogenase, the method of Pesce, McKay, and Stolzenbach was used. Crude cell extract was added to 300μ moles of potassium phosphate buffer (pH 7.0), 1.0μ moles of sodium pyruvate, and .35μ moles of NADH in a total volume of 3.0 ml. The reaction was initiated by the addition of sodium pyruvate, and the formation of nicotinamide adenine dinucleotide (NAD) from reduced NAD (NADH) followed spectrophotometrically at 340 nm on a Beckman model DU-2 ultraviolet spectrophotometer (Beckman Instruments Inc., Fullerton, California). See Table 3 for volumes.

Malate dehydrogenase was assayed according to Murphy and Kaplan, following the decrease in optical density at 340 nm caused by the formation of nicotinamide adenine dinucleotide (NAD) from

TABLE 3

Reagents for Enzyme Assays

LDH Enzyme Assay	
Component	ml
KPO_4 Buffer (pH 7.0, 0.1 M)	2.3
Na Pyruvate (Sigma Chem. Co., St. Louis, Mo.)	0.1
NADH (Sigma Chem. Co., St. Louis, Mo.)	0.1
Water (glass double-distilled)	0.4
Crude Enzyme Extract	0.1
TOTAL Volume Reaction Mixture	3.0

MDH Enzyme Assay	
Component	ml
KPO_4 Buffer (pH 7.5, 0.1 M)	2.3
Oxaloacetic Acid (Sigma Chem. Co., St. Louis, Mo.)	0.3
NADH (Sigma Chem. Co., St. Louis, Mo.)	0.3
Crude Enzyme Extract	0.1
TOTAL Volume Reaction Mixture	3.0

TABLE 3 (cont'd)

Glucose-6-Phosphate Enzyme Assay		
Component		ml
Tris-cl Buffer (pH 8.0, 1 M)		0.1
Glucose-6-Phosphate	(Sigma Chem. Co., St. Louis, Mo.)	0.1
TPN	(Sigma Chem. Co., St. Louis, Mo.)	0.1
$MgCl_2$ (0.1 M)	(J. T. Baker Chem. Co., Phillipsburg, N.J.)	0.1
Water	(glass double-distilled)	2.5
Crude Enzyme Extract		0.1
TOTAL Volume Reaction Mixture		3.0

reduced NAD (NADH). The assay mixture consisted of crude cell extract added to 300 μ moles of potassium phosphate buffer (pH 7.5), .33 μ moles of oxaloacetic acid and .13 μ moles of NADH, in a final volume of 3.0 ml. The reaction was initiated by the addition of oxaloacetic acid.

Glucose-6-phosphate dehydrogenase was assayed in accordance to Pandi and Cantino by following the increase in optical density at 340 nm caused by the formation of TPN from TPNH. The assay mixture contained crude cell extract added to 100 μ moles of tris-cl buffer (pH 8.0), 10 μ moles of $MgCl_2$, 2 μ moles of glucose-6-phosphate, and .15 μ moles of TPN, in a final volume of 3.0 ml. The reaction was initiated by the addition of glucose-6-phosphate. All reagents used were brought to 25 C for 10 minutes prior to use in a precision scientific water-bath (Precision Scientific Co., Inc., Chicago, Illinois). All pipetting for the assays was performed with Kimax, color-coded, safe-gard, tempered long tip, serological pipettes (Kimax-51 glass, No. 37039) (W. H. Curtain Co., Houston, Texas).

Disc-Gel Electrophoresis. Disc electrophoresis was performed on the plasmodial stage, the early sclerotial phase, and the completely differentiated sclerotial phase. The gels were prepared according to the procedure of O. Smithies (50) with slight

modifications. Pyrex glass was cut to a length of 80 mm with an inside diameter of 8 mm (W. H. Curtain Chemical Co., Houston, Texas). The ends were carefully fire-polished so as not to reduce the orifice size, and filed smooth. The pyrex tubes were then fitted with rubber stoppers, serum type, without sleeves (W. H. Curtain Chemical Co., No. 202-374, Houston, Texas), 11 mm in diameter and placed in a vertical position. The small-pore gel was then cast and allowed to solidify inside the pyrex glass tube at room temperature for 45 minutes. See Table 4 for compositions. A thin layer of water was placed over the top portion of the small-pore gel before exposure to room temperature to assure an even layering while the material hardens. This can be accomplished by the use of a 100 cc tuberculen syringe and 22 gauge needle (Becton, Dickinson and Company, Rutherford, N.J.), gently dripping 1 cc of double-distilled water down the side of the pyrex glass gel-tube. When the gel solidifies, the water may be gently shaken off. Seven tubes were used, with 3.1 ml of small-pore gel pipetted into each tube. The large-pore gel was then prepared and 0.3 ml pipetted into each tube, again layering with water to assure an even distribution of the material. Exposure to fluorescent light for 15 minutes facilitates the hardening process, after which time the water may be discarded. The rubber grommets are removed by pinching the ends upward slightly

so that the gel is not subjected to the force of a vacuum upon removal. The gel tubes are then moistened on the end with glycine buffer and carefully inserted into the upper reservoir stand, void of liquid content for the moment. The sample, a crude extract of the various phases of the organism, was prepared in an identical fashion to the enzyme assay procedure. A 0.1 ml sample of the crude extract added to 0.5 ml of 40% sucrose (Fisher Scientific Chemical Co., Fair Lawn, N.J.) was prepared and kept at 4 C until it was ready to be layered on the gel. Total protein of the prepared samples ranged from 70 ug to 350 ug, depending on the extract being used. Electrophoresis is performed in a vertical position, the gel containers attached to a glycine-tris buffer reservoir and the lower ends submerged in the buffer solution of a lower reservoir. Electrodes are placed in each reservoir and the polarity set so that the sample ions migrate toward the small-pore gel. A polystyrene housing apparatus, 6 inches in diameter and 2.5 inches deep secures the gels and provides the reservoir for the glycine buffer. Any extra holes not in use should be plugged with rubber grommets. The upper reservoir should be supported above the lower reservoir by a support ring attached to a ring stand. Electrode leads from the power supply, a Triplett 1000 ohms per amp power generator (Triplett Elec. Instrument Co., Model No. 327-PL, Bluffton, Ohio) are attached so that the anode (black lead)

is secured to the lower reservoir and the cathode (red lead) is attached to the upper reservoir. The leads are held firmly in place by a glass rod inserted securely in the reservoir; the rod is wrapped in aluminum foil to provide conductance.

The sample solution is now layered, gently, with a pasteur-pipette, equipped with a squeeze bulb on the end. Prior to the layering of the sample, all oxygen bubbles are removed from the reservoir and the surface of the gel-tubes. The heavier sample should layer smoothly on the top of the gel tube if the end of the pasteur-pipette is within reasonable proximity of its surface. After all seven tubes have been filled, the current is applied. The current is adjusted to about 2 to 5 milliamps per tube. The run is performed in the cold room at 10 C. On applying a voltage, a thin disc of Bromophenol Blue is seen to migrate into the gels. The Bromophenol Blue should be added to the upper glycine reservoir just before current initiation. It will overtake and pass the protein species which concentrate behind the dye as it migrates through the sample. Electrophoresis is performed until the tracking dye is almost to the bottom of the gel. At the completion of the run, the power is shut down. The electric leads from the power source are disconnected from the lower reservoir and from the upper reservoir containing the platinum electrode (platinum electrode, 5/8 inch diameter, 5 inch length, No. 195-321, W. H.

Curtain Co., Houston, Texas). The gel tubes are removed from the upper reservoir after the glycine buffer is decanted away. The gels are removed from the tubes with a 100 cc tuberculin syringe filled with glass, double-distilled water. The tip of the needle is just inserted at the small-pore end of the gel tube and a stream of water gently forced down the edge. The needle is then withdrawn with a slight pressure against the gel, stretching the gel so that it will protrude about 5 mm beyond the end of the tube. The needle is withdrawn completely, and introduced into the other end. A continuous rimming action is applied as the needle advances. The gel will then slip out of the tube. Each gel is placed in a test tube (covered with aluminum foil) containing 15.4 ml of a fixitive stain solution. See Table 4. The gels are studied sporadically to observe any reaction taking place, so as not to allow overstaining. If the specific enzyme being challenged was present in the sample, then appropriate band formation should be present. After a reaction has been observed, one must prevent overstaining of the gel by removing it from the fixative solution and emersing it into a 7.5% glacial acetic acid solution (Fisher Scientific Chem. Co., Fair Lawn, N.J.), and placing it in small test tubes fitted with screw caps. The gels are ready to be read on an Ansco color meter (Thomas A Schurty Co., Ansco, Binghampton, N.Y.).

TABLE 4

Stock Solutions, Reagents, and Preparations

Made for Disc-Gel Electrophoresis

Stock Solutions	

Solution A:

Component	Amount
1 N HCl, Reagent Grade (Fisher Chem. Co., Fair Lawn, N.J.)	48 ml
Tris, Ultra Pure, Enzyme Grade (Mann Laboratories, New York, N.Y.)	36.3 g
Temed (Eastman Kodak, Rochester, N.Y.)	0.23 ml
Water, glass double-distilled to pH 8.9	100 ml

Solution B:

Component	Amount
1 N HCl, Reagent Grade (Fisher Chem. Co., Fair Lawn, N.J.)	48 ml
Tris, Ultra Pure, Enzyme Grade (Mann Laboratories, New York, N.Y.)	5.98 g
Temed (Eastman Kodak, Rochester, N.Y.)	0.46 ml
Water, glass, double-distilled to pH 6.6	100 ml

TABLE 4 (cont'd)

Solution C:

Component		Amount
Acrylamide	(Eastman Kodak, No. 5521, Rochester, N.Y.)	28 g
Bis (N, N'-methylenebisacrylamide	(Eastman Kodak, No. 8383, Rochester, N.Y.)	0.735 g
Water, glass double-distilled to		100 ml

Solution D:

Component		Amount
Acrylamide	(Eastman Kodak, No. 5521, Rochester, N.Y.)	10 g
Bis (N, N'-methylenebisacrylamide	(Eastman Kodak, No. 8383, Rochester, N.Y.)	2.5 g
Water, glass double-distilled to		100 ml

Solution E:

Component		Amount
Riboflavin	(Eastman Kodak, No. 5181, Rochester, N.Y.)	4 g
Water, glass double-distilled to		100 ml

Solution F:

Component		Amount
Sucrose	(Fisher Chem. Co., Fair Lawn, N.J.)	40 g
Water, glass double-distilled to		100 ml

TABLE 4 (cont'd)

Solution G:

Component	Amount
Ammonium persulfate, reagent grade (Matheson Co., Los Angeles, Cal.)	.14 g
Water, glass double-distilled to	100 ml

Solution H:

Component	Amount
Tris (Ultra pure, Enzyme Grade) (Mann Laboratories, New York, N.Y.)	3 g
Glycine	14.4 g
Water, glass double-distilled to	100 ml

Solution J (Tracking Dye)

Bromophenol Blue Stain	.005%

Asundry

Solutions G and F should be used fresh.

Small Pore Gel (Solution No. 1):

MIX	
1 part A	To form the gel, combine
1 part C	1:1 with catalyst G
1 part water	Layer with water and let stand for 30-45 minutes.

TABLE 4 (cont'd)

Large Pore Gel (Solution No. 2):

MIX

1 part B — To form gel, expose to fluorescent light for 15 minutes

1 part D

1 part E

1 part F

Gel-Tube Preparation (Loading Sample):

Mix 0.5 ml of 40% Sucrose per 0.1 ml of crude extract. Gel-tubes should be chromic acid washed, rinsed, EDTA soaked, rinsed and Acetone dried. Use glass, double-distilled water for rinsing purposes.

NaDichromic Acid, saturated aqueous solution (W. H. Curtain Chem. Co., Houston, Texas)

EDTA (J. T. Baker Chem. Co., Phillipsburg, N.J.)

Gel-Tube Volumes:

Component	ml
Separating gel	3.1
Spacial gel	0.3
Sucrose (40%) and sample	0.6
TOTAL Volume	4.0

TABLE 4 (cont'd)

SPECIFIC STAINS FOR GEL-ELECTROPHORESIS

*Lactate Dehydrogenase: (Mix and incubate in the dark at 25 C)

Component	Amount
Tris-Cl Buffer, pH 7.5, 0.05 M	7.5 cc
Sodium dl-lactate, pH 7.5, 0.05 M (Sigma Chem. Co., St. Louis, Mo.)	3.0 cc
KCN, 0.06 M, pH 7.5 (Matheson Coleman & Bell, East Rutherford, N.J.)	1.25 cc
NBT (nitro-blue tetrazolium, 2 mg/ml) (Sigma Chem. Co., St. Louis, Mo.)	3.5 cc
DPN (diphosphopyridine nucleotide) (Sigma Chem. Co., St. Louis, Mo.)	5.0 mg
PMS (phenazine methosulfate, 2 mg/ml) Sigma Chem. Co., St. Louis, Mo.)	0.15 cc
TOTAL Volume	15.40 ml

*(51) with slight modification

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*Malate Dehydrogenase: (Mix and incubate in the dark at 25 C)

Component	Amount
Tris-Cl Buffer, pH 7.5, 0.05 M	7.5 cc
Sodium dl Malate, pH 7.5, 0.25 M (Sigma Chem. Co., St. Louis, Mo.)	3.0 cc
KCN, 0.06 M, pH 7.5 Matheson, Coleman, & Bell, East Rutherford, N.J.)	1.25 cc

TABLE 4 (cont'd)

	Amount
NBT nitro-blue tetrazolium, 2 mg/ml (Sigma Chem. Co., St. Louis, Mo.)	3.50 cc
DPN diphosphopyridine nucleotide (Sigma Chem. Co., St. Louis, Mo.)	5.0 mg
PMS phenazine methosulfate, 2 mg/ml (Sigma Chem. Co., St. Louis, Mo.)	0.15 cc
TOTAL Volume	15.40 ml

*(51) with slight modification.

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*Glucose-6-phosphate dehydrogenase (Mix & incubate in the dark at 25 C)

Component	Amount
Tris-Cl Buffer, pH 7.5, 0.05 M	7.5 cc
Glucose-6-phosphate, 0.1 M, pH 7.5 (Sigma Chem. Co., St. Louis, Mo.)	3.0 cc
KCN, 0.06 M, pH 7.5 (Matheson, Coleman, & Bell, East Rutherford, N.J.)	1.25 cc
NBT nitro-blue tetrazolium, 2 mg/ml (Sigma Chem. Co., St. Louis, Mo.)	3.50 cc
TPN triphosphopyridine nucleotide (Sigma Chem. Co., St. Louis, Mo.)	5.0 mg
PMS phenazine methosulfate, 2 mg/ml (Sigma Chem. Co., St. Louis, Mo.)	0.15 cc
TOTAL Volume	15.40 ml

*(51) with slight modification.

Amino Acid Analyses. Protein hydrolyzate, free amino acid analyses were performed on the plasmodia stage, the Phase I early sclerotial stage, and the completely differentiated stage, as well as the sporangium of the myxomycete P. flavicomum variety 1. The instrument used was a Phoenix Precision Instrument Company, Model K8000-C amino acid analyzer (Phoenix Prec. Instr. Co., Philadelphia, Pa.). The method used was that of Moore-Stein, protein hydrolyzate, accelerated amino acid analyses, with slight modifications (52).

Samples to be used in the analyses were prepared as follows:

Plasmodial Phase. The method of Kornberg, et al. (46) was used, with modifications. Log phase microplasmodia were harvested after 66 hours growth in the manner previously described. Protein determination and dry weights were sampled. All reagents were kept at 4 C throughout the procedure, especially following the breakage of the cells. The grinding procedure previously described for enzyme assay on plasmodium will be followed with slight modification. The cell pellet, placed in the cold porcelain mortar, was ground in the presence of 5% TCA (trichloracetic acid, Fisher Chem. Co., Fair Lawn, N.J.). The amount of 5% TCA used was usually 2 ml, and never varied more than 0.5 ml from preparation to preparation. The cells are ground and centrifuged following the procedure of the plasmodial phase enzyme assay. The supernatant was collected and the total volume noted.

A pH adjustment was made with 1 N NaOH, drop-by-drop, until the strong acidity of the TCA was overcome. The pH was taken to 7.5 and immediately dropped to pH 2.2 through the addition of 1 N HCl. The total volume of supernatant after pH adjustment was then recorded. The solution was removed from the ice-bath and frozen if an analysis was not to be run immediately. The frozen stock should be stored under a N_2 atmosphere and used within 5 days; otherwise a risk of deterioration is taken.

Phase I Sclerotia Preparation. The identical procedure used for preparing the plasmodial stock solution was used in preparing the Phase I stock sclerotium as well.

Phase II, Fully Differentiated Sclerotia. The identical procedure used in preparing both plasmodia and Phase I sclerotium shall be used in the preparation of the Phase II stock solution as well.

Preparation of the Spores. Spores of the organism were harvested and collected at various dates, immediately after fruiting experiments. They were kept frozen in double-distilled water to prevent germination until analyses could be performed. The spores were then prepared for analysis in the same manner as were the plasmodia and the sclerotial phases.

Analyses Procedure. Each phase of the myxomycete life

cycle was analyzed as a separate entity. The first phase to be analyzed was the plasmodial stage, then the early Phase I stage, followed by Phase II sclerotium and finally the spores. Each stage should be prepared and run within a span of 4 to 5 days, at maximum. Prior to the loading of the samples for the accelerated analyses, the machine must be prepared in the following manner: The acidic and basic columns (columns No. 1 and No. 4, respectively) must be cleared of debris from previous runs. This is accomplished by placing the machine on manual bypass and pumping a .4 N NaOH hydroxide, Brij-35, EDTA (dehydrate) solution through both columns. Pump No. 1 controls the NaOH wash, therefore the tygon lead to the No. 1 column must be interchanged with column No. 4 in order that it might be washed as well. Flush each column for 30 minutes. Replace the tygon leads to the proper columns. Pump buffer (pH 3.25) through column No. 1 for thirty minutes. The column manifold selector should be placed on No. 1 with the ninhydrin knob engaged. Next, pump column No. 4 with pH 5.28 buffer and change the manifold selector to No. 4. The ninhydrin selector remains unchanged. Column No. 4 should be pumped with buffer for 30 minutes as well. Check the reaction bath temperature. The reading should be 100 C. Engage the circulating water bath switch and allow the temperature to level at 50 C. The thermometer will register 50 C within 10 minutes after the initiation

of the circulating water pump. Remove the tygon leads to each column and check the pH to be assured that the proper buffers are in the respective lines. This procedure is a check as well to confirm that the manual bypass switch (for pumping NaOH) had been disengaged prior to pumping the buffers. Cap the ends of the tygon tubes with rubber stoppers and proceed to draw off the buffer inside both columns to a level of 1/8 inch over the top of the resin. Do not allow air to come in contact with the top of the resin, as this may result in the channeling of the amino acids. This procedure may be easily accomplished with the use of a 25 cc syringe fitted with a 5 inch teflon tubing, 3 mm inside diameter.

The columns are now ready to be loaded with the sample. Begin with column No. 1. Using a 1 ml pyrex Kimax, safe-gard, serological, tempered-tip pipette, exactly 1 ml of sample is loaded onto the top of the resin. The air line is secured in place on the column and the sample is forced into the resin to the original 1/8 inch level again with 40 lbs. of air-pressure. The line is removed and the column rinsed with 1 ml of pH 2.2 sodium citrate buffer. Allow the buffer to slowly wash down the sides of the column so as to collect as much sample as possible which may have been left behind. Again, apply 40 lbs. of air-pressure and stop the volume at 1/8 inch from the top of the resin. Remove the rubber cap from line No. 1 and

slowly rinse down the side of the column again with pH 3.25 buffer. Allow the column to fill to near capacity, then quickly insert the tygon fitting and screw it down firmly into place. Again, allow no air to enter the column. The acidic amino acids are now ready to be run. Repeat this identical procedure for the basic column, No. 4. Place the manifold selector on column No. 4, the shutdown timer should be disengaged. Set the buffer program time to 193 minutes. The "program to" switch should be in the down position. Photolamps should be sufficiently warmed. The temperature program time should be disengaged. The manual direction control switch should be in the up position. Place pump switches No. 2 and No. 3 in the automatic position. Circulating bath and reaction bath switches should be in the "on" position. The time-meter dial should be wound to flat zero, the recorder switch on automatic and the time-meter switch should read automatic as well. As the shut-down timer switch is engaged and set on 5.0 hours, the accelerated amino acid analysis procedure is initiated. The flow-rate lamp is switched on as well as the pressure recycling switch. The flow-rate is measured by injecting a small air bubble and timing it on the flow-meter gauge. The reading should be 15.0 seconds for the accelerated run.

A 15.0 second reading corresponds to a flow-rate of 120 ml/hr with the buffer and reagent pumps combined. Adjustments, if

necessary, are made at the pump itself by adjusting the micrometer dials just adjunct to the view-glass. At 7.0 minutes, the ninhydrin will enter the colorimeter and begin to register on the chart paper. At 9.5 minutes, the base line should be adjusted to zero. At 11.2 minutes the acidic amino acids will come off as one large peak. The reading should register 2.0 on the optical density scale for the .25 μ mole/ml standard with a variation of readings for the unknown samples. At precisely 133.0 minutes, the basic amino acid run will be completed. At this time, pump No. 2 is disengaged and pump No. 1 is switched to the automatic position. The manifold arrangement selector must be switched to read column No. 1. The acidic amino acid accelerated run is now in progress with 60 minutes showing on the buffer-program timer. The pH 3.25 buffer is now running through the acidic column, bringing the acidic amino acids off first, ahead of the neutrals. Midway through the proline amino acid peak a buffer change is initiated as time runs out on the buffer program timer switch. The pH now ascends slightly to 4.25, and the neutral amino acids are teased out. After completion of phenylalanine (279.0 minutes) the run is complete; however, one should extend the run out an extra 10 to 20 minutes in order to procure a lengthy base-line for calculation purposes. The machine should be flushed immediately after a run with .4N NaOH solution and rinsed completely with

buffer (both columns) as previously described. As well, the ninhydrin manifold selector switch should be positioned to "off" in order to prevent the backup of the reagent into the reaction bath coils when the machine is not in operation.

A complete list of all reagents needed for the efficient operation of the Phoenix Precision Instrument, Model K-8000-C amino acid analyzer is presented in Table 5.

A standard amino acid analysis was performed. All conditions were as previously described. The amino acid standard preparation was procured from Beckman Laboratories (Palo Alto, Calif.) Type 1 amino acid calibration mixture for amino acid analyzer, No. 31220, 2.500 $\pm$ .004 moles of the following l-form assymetric amino acids/ml: lysine, hystidine, ammonia, arginine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, half-cystine, valine, methionine, isoleucine, leucine, tyrosine, and phenylalanine. The solution was diluted 1:10 with pH 2.2 sodium citrate buffer and 1 ml, containing 0.25 μ moles of each amino acid, was used in the sample preparation to determine the standard curve. Calculations of the standard curve was by the "area integration by height-width method" (52). The equation expressing the relationship for the constant by which height-width is divided to give moles of amino acid/integrated area of each peak is:

TABLE 5

Reagents for the Preparation and Function of the Model K-8000-C, PPI, Amino Acid Analyzer (Moore-Stein, accelerated Protein Hydrolyzate Procedures

Sodium Citrate Buffer, pH 2.2 0.03	
Component	Amount
Citric Acid·H_2O (Fisher Scientific Chem. Co., Fair Lawn, N.J.)	21 g
NaOH 97% (Matheson, Coleman & Bell, East Rutherford, N.J.)	8.4 g
HCl, reagent grade, concentrated (Fisher Scientific Chem. Co., Fair Lawn, N.J.)	16 ml
Octanoic Acid (Matheson, Coleman & Bell, East Rutherford, N.J.)	0.1 ml
Thiodiglycol TG (Pierce Chem. Co., Rockford Ill.)	20 ml
Brij-35 solution, No. 20800 (Pierce Chem. Co., Rockford, Ill.)	2 ml
FINAL Volume (glass, distilled water) to	1 liter

Sodium Citrate Buffer: pH 3.25 0.01

Component	Amount
Citric Acid·H_2O (Fisher Scient. Chem. Co., Fair Lawn, N.J.)	840 g
NaOH 97% (Matheson, Coleman & Bell, East Rutherford, N.J.)	330 g

TABLE 5 (cont'd)

Component	Amount
HCl, reagent grade, concentrated (Fisher Scient. Chem. Co., Fair Lawn, N.J.)	426 g
Octanoic Acid(Matheson, Coleman & Bell, East Rutherford,N.J.)	4.0 ml
Brij-35 solution No. 20800 (Pierce Chem. Co., Rockford, Ill.)	80 ml
Thiodiglycol TG (Pierce Chem. Co., Rockford, Ill.)	200 ml
*n-propanol	1200 ml
FINAL Volume (Glass, distilled water) to	40 liters

*To improve resolution in the aspartic acid, threonine, serine area as well as to facilitate separation between serine, threonine, n-propanol was added 3% to v/v (53).

Sodium Citrate Buffer, pH 4.25 0.02

Component	Amount
Citric Acid·H_2O (Fisher Scient. Chem. Co.,Fair Lawn, N.J.)	840 g
NaOH 97% (Matheson, Coleman & Bell, East Rutherford,N.J.)	330 g
HCl, reagent grade, concentrated (Fisher Scient. Chem. Co., Fair Lawn, N.J.)	188 ml
Octanoic Acid(Matheson, Coleman & Bell,East Rutherford, N.J.)	4.0 ml
Thiodiglycol TG (Pierce Chem. Co., Rockford, Ill.)	200 ml
Brij-35 solution No 20,800 (Pierce Chem. Co., Rockford, Ill.)	80 ml
FINAL Volume (glass, distilled water) to	40 liters

TABLE 5 (cont'd)

Sodium Citrate Buffer: pH 5.28 0.02

Component	Amount
Citric Acid·H_2O (Fisher Scient. Chem.Co.,Fair Lawn, N.J.)	491 g
NaOH 97% (Matheson, Coleman & Bell, East Rutherford, N.J.)	288 g
HCl, reagent grade, concentrated (Fisher Scient. Chem. Co., Fair Lawn, N.J.)	136 ml
Octanoic Acid (Matheson, Coleman & Bell, East Rutherford, N.J.)	2.0 ml
Brij-35 solution No. 20,800 (Pierce Chemical Co., Rockford, Ill.)	40. ml
FINAL Volume (glass, distilled water) to	20 liter

.4N NaOH Solution

NaOH Pellets (Matheson, Coleman & Bell, East Rutherford, N.J.)	16 g
*Brij-35 solution No. 20,800 (Pierce Chem. Co., Rockford, Ill.)	5 ml
EDTA	1 g
Water (glass distilled) to	1 liter

*50 g Brij-35 dissolved in 100 ml hot, de-ionized water. If it comes out of solution at 25 C, the amount of water should be increased to 150 ml, and 1.35 times the amount should be used in all reagents.

NOTE: In preparing the sodium citrate buffers, dissolve the citric acid first, then the NaOH, in 5 liters of water. Add the HCl, the Octanoic Acid, TG, and Brij-35. Cool to room temperature, after which fill the bottle to volume. Mix the buffer

TABLE 5 (cont'd)

thoroughly in 10 additional liters of water. Bring to full volume. When the solution temperature is equal to the ambient temperature ($\pm$ 0.5 C) take a pH reading. Let it stand overnight, if necessary. If an adjustment is necessary, the addition of 1 ml of 50% NaOH or 2 ml of concentrated HCl causes a change of about 0.01 pH units in any of the 40 liter or 20 liter quantities of buffers prepared.

Ninhydrin Reagent.

In order to efficiently and accurately prepare the ninhydrin reagent for amino acid analysis, the procedure outlined in the Liquid Chromatography Handbook must be followed explicitly. Preparation and transfer of this reagent to the analyzer is perhaps the most important step in any of the analyses procedures. See Liquid Chromatography Handbook (52), section on preparation of reagents, pages 32-34.

$$\mu \text{ Moles} = \frac{H \times W}{C_{hw}}$$

where μ moles is the concentration, H x W the peak area, and C_{HW} is the HW constant.

The constant, H x W per μ mole is a function of the color yield of the given amino acid in the ninhydrin reaction (52). Integration by the net total absorbance method may be used as well (52). Calculations for the amino acid data were performed with the aid of a Monroe 8F-213 monro-matic calculator (Monroe International, Inc., Orange, N.J.).

Analytical Methods. To calculate growth of the microplasmodia as well as sclerotial and spore yields, two parameters were taken, protein and dry-weight measures. The microplasmodia, the two sclerotial phases and the spores were all brought to total volume in basal salts, and 1.0 and 5.0 ml aliquots removed. A protein determination was performed on the 1.0 ml aliquot, while dry-weight measures were taken on the 5.0 ml sample.

The 1.0 ml aliquot was centrifuged at 4 C for 10 minutes at 15,000 rev/min (27,000 x g) in a Servall Refrigerated automatic centrifuge. Corning 15 ml centrifuge tubes were used for all centrifugations. The supernatant was poured off and 1.0 ml of 10% trichloroacetic acid (w/v) and 1.0 ml of acetone (Matheson, Coleman, & Bell;

Easthaven, N.J.) was added to each tube. The pellet was resuspended by vibrating on a Matheson Super Mixer No. 601001-05, (Matheson, Coleman & Bell, Easthaven, N.J.). The supernatant was poured off and the pellet resuspended in 1.0-3.0 ml of 0.4N NaOH, depending on the size of the pellet. The protein content was then determined by the method of Lowry, et al. See Table 6. One part of a 0.1% $CaSO_4 \cdot 5H_2O$ (w/v) was mixed with nine parts of the alkaline tartrate solution. Five ml of this solution was added to 0.5 ml of the resuspended pellet (in 0.4N NaOH) and allowed to stand for 10 minutes. One-half ml of Folin Ciocalteu Phenol reagent (Anderson Laboratories, Fort Wort, Texas), was mixed 1:1 with water, and added to the solution. Thirty minutes was allowed for full color development. The optical density was read at 700 mμ on a Beckman DU-2 spectrographotometer.

The standard for the protein determination was prepared by dissolving 0.050 g of Bovine Serum Albumin (Armour Pharmaceutical Co., Kankakee, Illinois) in 20 ml of 0.02N HCl. The optical density of this solution was determined at 278 mμ . According to the following extinction coefficient, the actual amount of BSA in solution was determined (55).

$$AS = 0.64 \text{ liter} \cdot g^{-1} \cdot cm^{-1}$$

By dividing the optical density at 278 mμ by 0.64, the actual amount

TABLE 6

Reagents for the Lowry Protein Determination

Bovine Serum Albumin Standard:

Component	Amount
Bovine Serum Albumin	0.050 g
HCl, 0.02N, reagent grade	20 ml

Alkaline Tartrate:

Component	Amount
NaOH	4.4
Na_2CO_3 (Matheson, Coleman & Bell, East Rutherford, N.J.)	44.
$Na_2C_4H_4O_6 \cdot 2H_2O$ (Malinckrodt Chemical Co., St. Louis, Mo.)	0.4 g

Copper Sulfate:

Component	Amount
$CuSO_4$ (J. T. Baker Chem. Co., Phillipsburg, N.J.)	0.1 g
Water (glass, double-distilled) to	100 ml

Copper Sulfate--Alkaline Tartrate:

Component	Amount
Copper Sulfate	1 part
Alkaline tartrate	9 parts

of BSA per ml is determined. A standard curve was then determined for the BSA solution. Protein measures on the plasmodial phase, Phase I and Phase II sclerotium, and the spores were taken in this manner, to be used in the enzyme assays and amino acid analyses, as well as an index of growth.

Dry-weight determinations were performed on all the various morphogenetic phases of the myxomycete, P. flavicomum variety 1. A 5.0 ml aliquot of each phase was removed from a known volume and placed in an aluminum foil weighing dish (No. 079-053, W. H. Curtain Co., Houston, Texas). The weighing dish had been previously heated to 100 C for 2 hours in a Precision Thelco drying oven (No. 022-822, W. H. Curtain Co., Houston, Texas), after which time it was brought to 30 C inside the oven, then removed with forceps and placed in a dessicator (Pyrex, No. 076-653, W. H. Curtain Co., Houston, Texas), filled with a suitable amount of dessicant (Drierite, $CaSO_4$, W. A. Hammond Drierite Co., Xenia, Ohio). A vacuum was pulled on the container and the weighing dish allowed to stand overnight at room temperature. At the time of the analysis, the vacuum was released on the dessicator, and the weighing dish quickly removed with forceps and placed on a Sartorius Analytical balance (Hamburg, Germany) and weighed. The 5.0 ml Aliquot was pipetted at room temperature into the weighing dish and then the dish returned to the hot-air oven. The

weighing dish and cells were completely dessicated at 110 C, overnight. The pan was returned to the dessicator after being completely dried in the hot-air oven, placed under vacuum pressure and left overnight at room temperature. The following morning the vacuum was released, the pan removed with forceps and an accurate weight determined on the dessicated cells.

Statistical Analysis. The enzyme assay analyses were computed over eight trials. A statistical analysis was performed on each of these eight trials as to their mean, standard error of their mean, confidence interval at the .95 and .99 levels of rejection, standard deviation, standard error of the standard deviation, and confidence interval level of rejection for a standard deviation at the .95 and .99 levels (56). As well, a correlation coefficient analysis was performed on the net data values obtained from the eight assay trials on each morphogenetic phase of the organism, except the spores. The raw data was fed into a Univac No. 1108 Exec. Vers. 267098115709 auxillary computer with a print out matrix in a free format upon specific input code request. The code used was TF and the available analysis was correlation of matrix.

Miscellaneous. All glassware was handwashed in detergent solution, rinsed in tap water, and then rinsed in double-distilled water and air dried. Pipettes were cleaned in a sulfuric acid sodium dichromate cleaning solution and rinsed in tap water, then glass

double-distilled water. All reagents used in this research were of reagent grade unless otherwise specified.

RESULTS

A varied array of fruiting percentages were found when testing the effects of different sporulation media on the myxomycete, P. flavicomum variety 1. The most consistent fruiting percentages are reported in Figure 1, where glass, double-distilled water, pH 4.0 was used as the substrate. The criterion for judging a plate positive was if at least 50% of the available plasmodium had fruited. Scanty indications of fruiting bodies on an experimental plate were judged as negative. The percentages found ranged from a low of 28% to a high of 92%, with a mean value of 71% over sixteen trials. Trial No. 17 represents a control group subjected to the identical procedure as the other plates, only never given any light.

Salts-niacin solution was also used as a fruiting medium. Over a period of 6 months, 10 trials were performed. The results are shown in Figure 2. The percentages ranged from a low of 5% to a high of 50%, with an average value of 24%. Trial No. 11 consisted of plates subjected to the identical conditions as the other experimental plates, only never receiving any light. There was no observable difference between the filter sterilized preparation and the autoclaved preparation since neither of the media appeared to

FIGURE 1

Percent fruiting of P. flavicomum variety 1 over sixteen trials, using pH 4.0 glass-distilled water as a substrate.

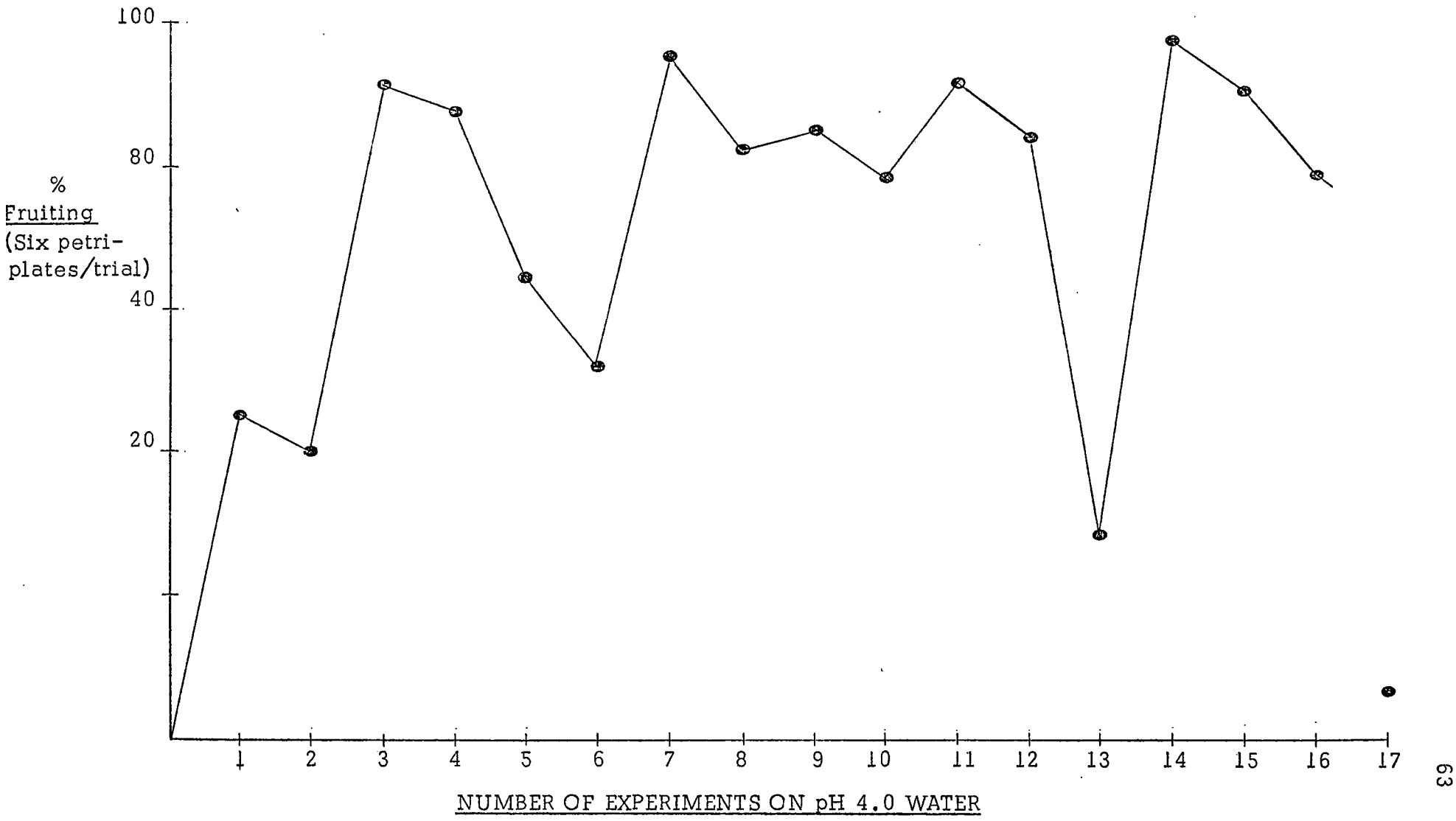
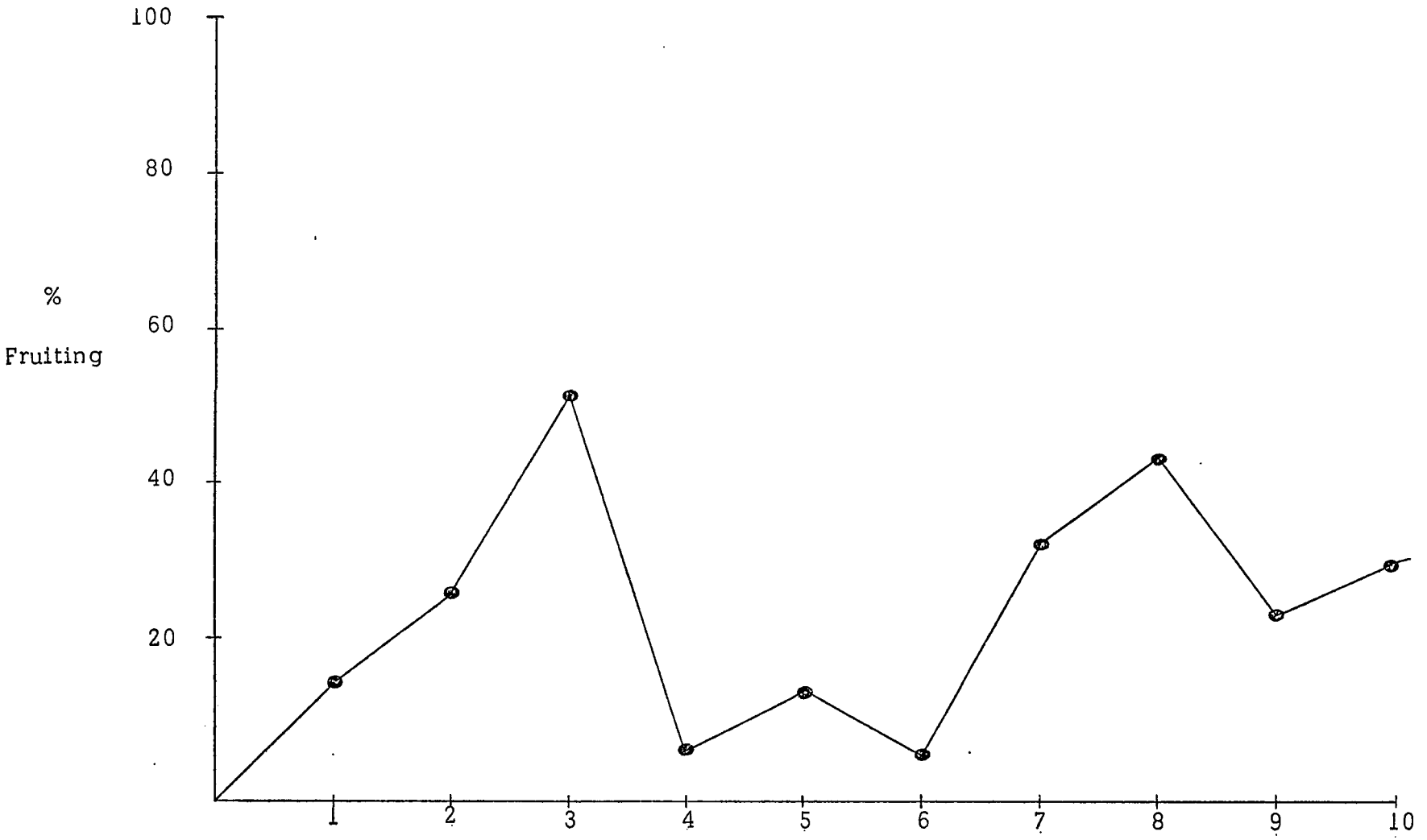

100
80
%
Fruiting
(Six petri-
plates/trial)
40
20
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
NUMBER OF EXPERIMENTS ON pH 4.0 WATER

FIGURE 2

Percent fruiting of P. flavicomum variety 1 over ten trials, using salts-niacin solution, pH 4.0 as a substrate. Six petri-plates per trial.

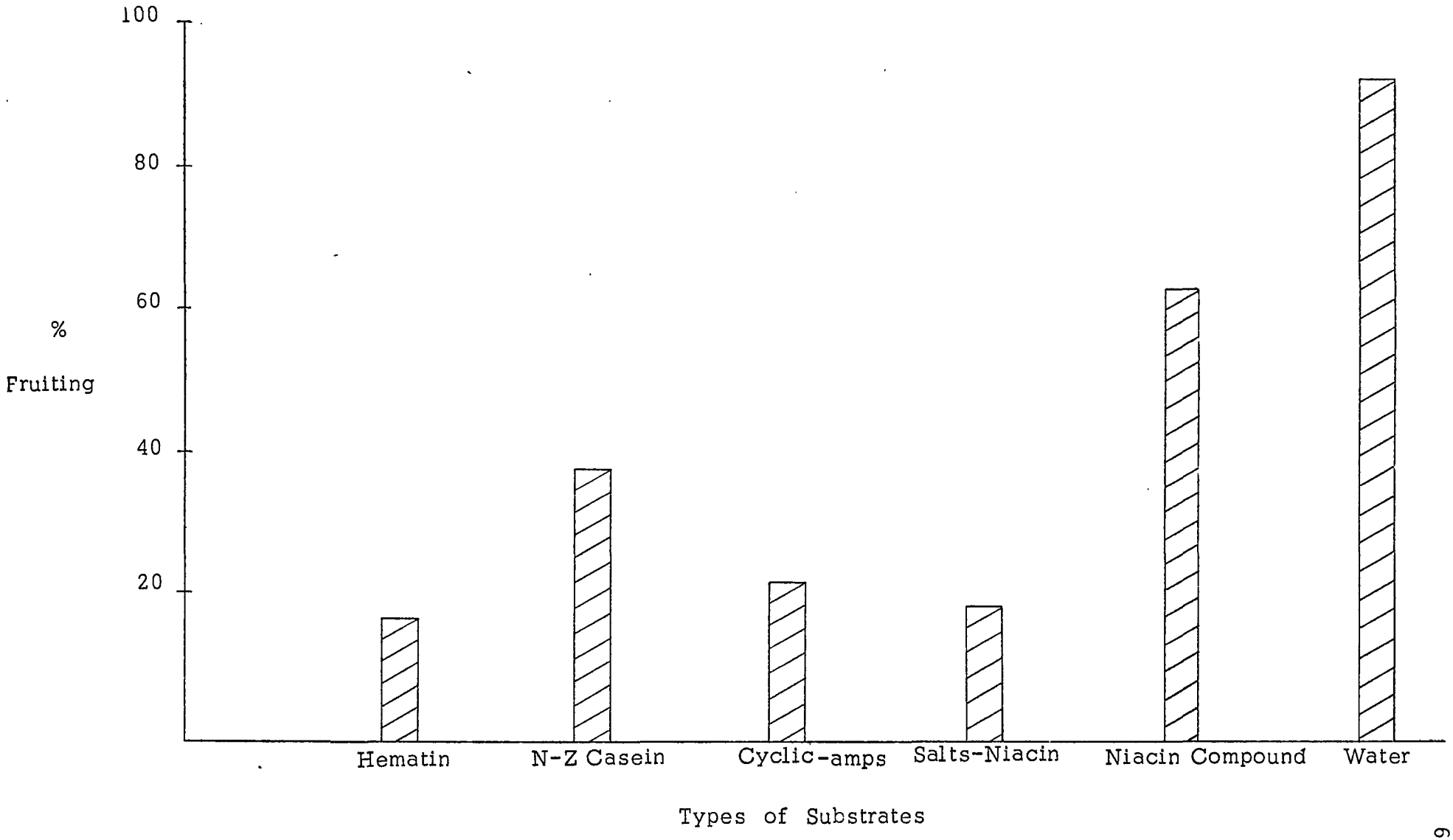

100
80
60
40
20
%
Fruiting
1
2
3
4
5
6
7
8
9
10
Number of Experiments on Salts - Niacin Substrate

facilitate fruiting over that of the pH 4.0 water medium. Deleting the basal salts and using only the niacin compounds in pH 4.0 water gave results similar to, but still not as effective as the water substrate alone. Basal salts and water, pH 4.0 was relatively poor as a sporulation media.

Other sporulation media were tried as well. N-Z casein hydrolysate produced only sporadic, unpredictable fruiting, as did the cycle AMP preparations, the hematin solution, and the catechol solution. In one particular experiment consisting of 18 petri-plates the following protocol was set up. Three petri-plates consisted of hematin, three of N-Z casein, three with cyclic AMP, three with salts-niacin, three with niacin compound and water, and three with water only. All were adjusted to pH 4.0 and subjected to identical preparative conditions. The fruiting percentage results can be seen in Figure 3. From the graph, the water plates are observed to fruit in the highest percentage, followed by the niacin compounds. The remainder of the plates follow closely, percentage-wise. A control group consisted of two petri-plates, placed with the experimental plates, only shielded during the illumination process with an aluminum foil cover, adjusted so that air would circulate but allow no light to enter. The controls did not fruit at all. Fruiting percentages per trials run show the pH 4.0 water medium to provide the best substrate for sporulation.

FIGURE 3

Percent fruiting from one experiment using a variety of substrates.


100
80
60
40
20
%
Fruiting
Hematin
N-Z Casein
Cyclic-amps
Salts-Niacin
Niacin Compound
Water
Types of Substrates

The various colored, incandescent floodlamps provided little in the way of facilitating fruiting. Three individual trials were performed with each lamp, and only the blue lamp ever produced spores (20% fruiting). The red lamp and the yellow lamp were poor in their sporulative capacity.

Fluorescent lighting was found to be not as effective in producing fruiting bodies as the incandescent, tungsten lamp source, although the fluorescent lamps were a better source for sporulation than were the floodlamps. The most reliable illumination system was that of the incandescent tungsten bulb assembly. The amount of time necessary for light exposure should be 4 hours at a minimum, and 24 hours at a maximum. Cultures were found to fruit best in this range, although a mean value of 6 hours of illumination was most often used. Cultures had a tendency to exhibit lower fruiting percentages during the months between December through March, with the highest percentages being registered in mid-Spring through early Fall.

A crude acetone extract of the pigment of P. flavicomum variety 1 was observed spectrophotometrically at various wavelengths. The optical density values are quite divorced from the values obtained by Gray using P. polycephalum (16). See Figure 4. The greatest optical densities for P. flavicomum variety 1 were in the vicinity of

FIGURE 4

Crude acetone extract of the myxomycete P. flavicomum variety 1.


OPTICAL DENSITY
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
190 200 210 220 230 240 250 260 270 280 290 300 310 320 330 340 350 360 370 380 390 400 410 420 430 440 450 460 470 480 490 500 510 520 530 540 550 560 570 580
WAVELENGTH -- ANGSTROM UNITS

210 nm and 360-420 nm, while Gray reports maximums at 430 nm.

RESULTS OF ENZYME ASSAYS

The statistical mean, standard deviation, confidence intervals, and correctional values for the specific activity values of the plasmodia and both differentiating phases of the sclerotium of the myxomycete, P. flavicomum variety 1 are cited in a summary chart in Table 7. Each value represents a statistical computation over eight trials. For example, the mean value of the LDH plasmodial phase was found to be .3690. This figure represents the average specific activity value of the LDH enzyme, plasmodial phase, taken from eight individual experiments. The remaining categories are statistical validation that the .3690 value found over eight trials for the LDH enzyme assay is indeed a reliable representation on which one may depend. Specific activity values were obtained by dividing total units and total mg of protein found in original cell pellet. See Table 8.

INTERPRETATION OF THE DATA CHART

In studying the data chart to be presented one may interpret a variety of disclosures as to the meaning and significance of the data at hand.

TABLE 7

Summary chart of data obtained from enzyme assays on the plasmodial stage, the Phase I stage, and the Phase II stage of the myxomycete P. flavicomum variety 1. Lactate dehydrogenase (LDH), malate dehydrogenase (MDH), glucose-6-phosphate dehydrogenase (G-6-PO_4).

	N	M	Gm	C.I. .95	C.I. .99	S
LDH Plasmodia	8	.3690	.1350	.1044-.6336	.0207-.7173	.3818
LDH Early Sclerota	8	.5220	.1990	.1320-.9100	.0086-1.0354	-.7640
LDH Late Sclerota	8	.1258	.052	.0239-.2271	-.0083-.2599	.466
MDH Plasmodia	8	3.82	1.51	.8604-6.7795	-.0758-7.7158	4.3
MDH Phase I	8	8.835	3.716	1.5517-16.1183	-.7522-18.4222	10.5
G-6-PO_4 Plasmodia	8	.1629	.0640	.0375-.2883	-.0022-.3280	.4306
G-6-PO_4 Phase I	8	.3121	.121	.0750-.5492	0.0000-.6242	.341
G-6-PO_4 Phase II	8	.1692	.071	.0301-.3083	-.0139-.3523	.200

SUMMARY CHART OF DATA FROM
ENZYME ASSAYS ON P. FLAVICOMUM VARIETY 1

TABLE 7 (cont'd).

	σ_s	C.I. .95	C.I. .99	2/3 Data Range	X_2	*Accept or Reject
LDH Plasmodia	.0954	.1948-.5688	.1357-.6269	-.0128 to .7508	41.8	R
LDH Early Sclerota	.1410	.2877-.8403	.2003-.9277	-.0420 to 1.080	36.2	R
LDH Late Sclerota	.1165	.2377-.6943	.1544-.7665	-.3402 to .5918	29.5	R
MDH Plasmodia	1.075	2.2 - 6.5	1.5 - .6.1	-.4800 to 8.12	23.6	R
MDH Phase I	2.63	5.3652-15.6748	3.7346-17.3054	-1.685 to 19.355	18.2	R
MDH Phase II	.600	1.2240-3.5760	.8520-3.9480	-.3571 to 4.4429	18.8	R
G-6-PO_4 Plasmodia	.1076	.3198-.5414	.1530-.7082	-.2677 to .5935	29.6	R
G-6-PO_4 Phase I	.0852	.1741-.5079	.1212-.5608	-.0289 to .6531	31.8	R
G-6-PO_4 Phase II	.050	.1020-.2980	.0710-.3290	-.0308 to .3692	18.2	R

*Accept or Reject Hypothesis Due to Chance Alone.

Begin by interpreting the mean and its standard error, represented by the symbols M and σ_m. The mean itself is easily calculated. Using LDH Plasmodia Phase as an example there is a mean value of .3690 over eight trials. This value may or may not represent the true mean. In fact, it may be in error by as much as thirty-six per cent, represented by the value of .1350 (σ_m). From the data values for the mean and its standard error, confidence levels are established in which the mean should fall a certain percentage of the time. Observing the chart, one finds that at a confidence level of .95 the mean should be between the values of .1004 and .6336, nineteen out of twenty trials. Likewise, the mean should fall between the values of .0207 and .7173, ninety-nine times out of one hundred trials at the .99 level of significance. Thus, the sample mean is established and the amount of error contained in that sample mean is based on chance and the number of trials observed.

The standard deviation reveals much concerning the distribution of the data in terms of the percentages of cases included within the range from one standard deviation below the mean to one standard deviation above the mean. Using LDH, plasmodia phase, one computes a standard deviation of .3818. As with the mean, this figure is also in error by 24%. One uses this error (σ_δ) to establish confidence intervals, just as in the values for the mean. The values

established here are between .1948 and .5688 for the .95 level of significance and .1367 and .6269 for the .99 level of significance. Therefore, one should expect the standard deviation to follow these interval limits as per number of trials and on the basis of chance, just as the mean followed them. Since the standard deviation is in fact an indicator of the amount of dispersion in a distribution, one should be able to set up data limits using the mean and the σ_6 of the S value observed. When such computations are performed, one finds limit values of -.0128 through .7508. These values represent the cut-off points for a normal distribution (2/3 of a bell-shaped curve) based on the data used. Any data lying outside these limits should be discarded as it would not be statistically valid. The LDH plasmodial phase has no specific activity values which were not randomly distributed.

In essence, every time a specific activity is calculated on the LDH plasmodia phase, one should expect to obtain a value between .0207 and .7173, ninety-nine times out of one hundred. The values obtained should be close to the sample mean of .3690 by an error percentage of 36% at maximum. The remainder of the data values discussed merely substantiates that the specific activity values used are indeed acceptable within the realms of a normal distribution.

Computer analyses were determined on the specific activity

TABLE 8

Specific Activity Calculation Example.

TABLE 8

Specific Activity Calculation Example

PREPARATION OF SAMPLE:

Used 3 ml of .05 M Tris-NCl buffer to bring crude extract to solution.

Used 1 ml of the crude extract for the 3 ml reaction mixture. (Made no further dilution.)

Observed a .052 OD/ml/min 340 mm drop (over a 3-minute period).

CALCULATION:

1.	.052	Average O.D. drop and 340 mm/1 min.
2.	3 ml	Volume of total reaction mixture.
3.	.1 ml	Volume used for reaction (sample) undiluted.
4.	1	Dilution factor for any subsequent dilutions.
5.	6.22	Constant
6.	Total protein	200 mg

$$\frac{\text{x units}}{1\text{ ml}} = \frac{.052 \quad (3\text{ ml total Rx. Mix.})\ (10)\ (1).}{6.22}$$

TABLE 8 (cont'd)

$$\frac{\text{x units}}{\text{1 ml}} = \frac{\text{.25 units}}{\text{1 ml}}$$

Total units is
.25 units x 3 = .75 units.

Total Protein	200 mg
$\frac{\text{Total units}}{\text{Total Protein}}$	$\frac{.75}{200}$

Total Specific Activity = .0037

values for the plasmodial phase, Phase I sclerotium, and the fully differentiated sclerotium. A correlation coefficient was determined to observe the relationship between each of the morphogenetic stages. The results are shown in Table 9. The remaining correlation coefficients for the malate dehydrogenase enzyme assay and glucose-6-phosphate dehydrogenase assay are shown in Table 10.

RESULTS OF GEL-ELECTROPHORESIS

Disc-gel electrophoresis was run on the plasmodium, the Phase I sclerotium, and the Phase II sclerotium. In all cases the specific stain tested was positive and typical. LDH gels were studied for the plasmodium, Phase I and Phase II sclerotium, with no observable differences in the integrity of the bands from one phase to the other. MDH gels revealed two bands, the mitochondrial form of the enzyme being the smaller of the two, and located ahead of the larger band. There was no change in the characteristic pattern of glucose-6-phosphate bands over the three phases tested.

RESULTS OF AMINO ACID ANALYSES

The results of the amino acid analyses of the acid soluble pools are shown in Tables 11, 12, 13, and 14.

Total protein measures using the Lowry technique were performed on

TABLE 9

An Example of the Correlation Coefficient Matrix for the LDH specific Activity Values on the Various Morphogenetic Stages of the Myxomycete, *P. flavicomum* variety 1.

Lactate Dehydrogenase		
Trials	Row 1 Plasmodium	Row II Phase I
(1)	.365	.500
(2)	.352	.610
(3)	.410	.560
(4)	.324	.523
(5)	.465	.490
(6)	.306	.348
(7)	.348	.549
(8)	.382	.596

CORRELATION MATRIX

Row I
1.000 .289

Row II
.289 1.000

Correlation Coefficient is .289.

TABLE 9 (cont'd)

Lactose Dehydrogenase		
Trials	Row I Phase I	Row II Phase II
(1)	.500	.132
(2)	.610	.204
(3)	.560	.080
(4)	.523	.077
(5)	.490	.196
(6)	.348	.175
(7)	.549	.039
(8)	.596	.103

CORRELATION MATRIX

Row I	
1.000	-.282
Row II	
-.282	1.000

Correlation Coefficient is -.282.

TABLE 9 (cont'd)

Trials	Row I	Row II
(1)	.365	.132
(2)	.352	.204
(3)	.410	.080
(4)	.324	.074
(5)	.465	.196
(6)	.306	.175
(7)	.348	.039
(8)	.382	.103

CORRELATION MATRIX

Row I	
1.000	.198
Row II	
.198	1.000

Correlation Coefficient is .198.

TABLE 10

Correlation matrix values for malate dehydrogenase and glucose-6-phosphate dehydrogenase.

Malate Dehydrogenase	
Plasmodium, Phase I	.307
Phase I, Phase II	.197
Phase II, P asmodium	-.016

Glucose-6-phosphate dehydrogenase	
Plasmodium, Phase I	.106
Phase I, Phase II	.208
Phase II, Plasmodium	-.101

TABLE 11

Amino Acid Analysis of the Acid Soluble Pool of the Plasmodial Myxomycete, P. flavicomum Variety 1.

Amino Acid	Moles/ 100 moles of Amino Acid	Moles of amino acid/100 moles of Amino Acid
Aspartic acid	1.55	1.73
Threonine-Serine complex	15.81	17.66
Glutamic acid	9.26	10.35
Proline	25.20	28.15
Glycine	1.76	1.97
Alanine	8.47	9.46
Valine	12.90	14.40
Methionine	1.08	1.21
Isoleucine	3.18	3.55
Leucine	4.85	5.42
Tyrosine	.40	.45
Phenylalanine	1.62	1.81
Ammonia	10.51	----
Lysine	2.91	3.25
Histidine	.22	.25
Arginine	.28	.31

TABLE 12

Amino Acid Analyses of the Acid Soluble Pool of the Phase I Sclerotium of the Myxomycete, P. flavicomum Variety 1.

Amino Acid	Moles/100 moles of Amino Acid	Moles of Amino Acid/100 moles of Amino Acid
Aspartic acid	3.16	4.10
Threonine-Serine complex	30.37	37.50
Glutamic acid	8.66	10.70
Proline	3.33	4.10
Glycine	3.90	4.80
Alanine	6.74	8.30
Valine	6.53	8.10
Methionine	1.63	2.01
Isoleucine	3.64	4.45
Leucine	6.55	8.07
Tyrosine	.92	1.14
Phenylalanine	1.01	1.23
Ammonia	19.10	----
Lysine	3.67	4.52
Histidine	.30	.37
Arginine	.49	.61

TABLE 13

Amino Acid Analyses of the Acid Soluble Pool of the Phase II Sclerotium of the Myxomycete, P. flavicomum Variety 1.

Amino Acid	Moles/100 moles of Amino Acid	Moles of Amino Acid/100 Moles of Amino Acid
Aspartic acid	2.34	4.54
Threonine-Serine Complex	9.36	18.16
Glutamic acid	3.78	7.34
Proline	4.32	8.38
Glycine	5.60	10.87
Alanine	7.27	14.11
Valine	5.64	10.95
Methionine	.61	1.18
Isoleucine	2.66	5.16
Leucine	3.59	6.97
Tyrosine	.56	1.08
Phenylalanine	.68	1.32
Ammonia	48.47	----
Lysine	2.31	4.48
Histidine	1.93	3.75
Arginine	.88	1.71

TABLE 14

Amino Acid Analyses of the Acid Soluble Pool of the Spores of the Myxomycete, P. flavicomum Variety 1.

Amino Acid	Moles/100 Moles of Amino Acid	Moles of Amino Acid/100 Moles of Amino Acid
Aspartic Acid	1.10	4.33
Threonine-Serine Complex	2.67	10.50
Glutamic Acid	1.77	6.97
Proline	2.30	9.10
Glycine	2.38	9.37
Alanine	3.16	12.44
Valine	3.59	14.12
Methionine	.33	1.30
Isoleucine	1.06	4.17
Leucine	1.82	7.17
Tyrosine	.23	.90
Phenylalanine	.38	1.50
Ammonia	74.60	----
Lysine	2.92	11.50
Histidine	.97	3.80
Arginine	.72	2.83

plasmodial phase, Phase I sclerotium, and the completely differentiated Phase II sclerotium. The results are shown in Table 15. The values observed represent a decrease over time, as the organism enters the fully sclerotized state from the plasmodial stage. Dry weight measures are represented as well, for the various morphogenetic phases of the organism.

TABLE 15

Lowry Protein Determinations and Dry-weight Measures on the Plasmodium, Phase I and Phase II sclerotium of the Myxomycete, P. flavicomum Variety 1.

Total Volume in ml	Dry-weight in g/5 ml sample	Total Dry-weight in g	Total Protein in mg	Percent of Protein per total Dry-weight
Plasmodial Phase				
225	.0805	3.623	248.107	6.9%
Phase I				
225	.0486	2.187	188.116	8.6%
Phase II				
225	.0388	1.746	130.536	7.46%
Spores				
26	.0025g/1ml	.0650g	18.41	27.7

DISCUSSION

The myxomycete, P. flavicomum variety 1, has been shown to sporulate on pH 4.0 water substrate more readily than on a variety of other sporulation media provided it. Perhaps the least complex of media, the water substrate, provides the most efficient means of suitably starving the organism and priming it for sporulation. Some of the other media, cyclic AMP and the niacin compounds for example, should have provided a reasonable substrate on which sporulation might ensue, since these compounds are supported by other researchers to be strong in their effect as acrasins (56), and as precursory material triggering the fruiting process (32). Since these materials provided little in the way of producing adequate fruiting, they shall be assumed to be ineffective as a fruiting media on the system used in this research. This does not discount the fact that on other sporulation systems, at different concentrations, these compounds might well prevail over the water substrate to which they played a secondary role in overall fruiting percentages. However, the water substrate was shown to far exceed any of the other fruiting media used in this research, and should therefore be considered the most efficient means of producing fruiting bodies.

The tungsten lamp illumination system was selected as the best due to its ability to produce fruiting bodies more reliably and with greater percentages than any of the other lighting systems tested. The tungsten lamp emits a color spectra primarily in the near red region, with adequate amounts of far blue as well. These wavelength regions coincide fairly well with the optical density readings found in the crude, acetone extract of P. flavicomum, however they lend no supportive evidence to the postulate of the plasmodial pigment being instrumental as a photoreceptor. Therefore, with all environmental parameters in check (30) the tungsten lamp system and pH 4.0 water substrate provides an adequate means of achieving sporulation in the myxomycete, P. flavicomum variety 1.

Upon observing the data and statistical manipulations of the specific activities of the organism, P. flavicomum variety 1, certain inferences may be made.

The data has been shown to be statistically sound. All of the specific activities used fell within the realm of a normal distribution pattern and needed not be discarded. The sample means calculated for this assay would be computed again in another assay within a certain per cent error (fluctuation). This new mean would be accurate within certain interval limits contingent to the error margin found, and would be expected to appear ninety-nine times out of

one hundred trials. In essence, if I ran the experiment again I would expect the same results to show up on the basis of statistical predictability.

The total protein content is an integral facet of the specific activity value at which one finally arrives. Any degree of fluctuation here would certainly prove to be a governing influence on the final S. A. value achieved. The protein content of the cell appears to be decreasing as the cell moves from a vegetative to a more differentiated state. If this trend of decreasing protein content in the cell were the only observable phenomenen taking place during the differentiation process then surely one would observe a gradual increase in the S. A. values over time. This is not the case; therefore, other intervening factors, surreptitiously disguised, have to be accounted for.

The question arises as to why if the total protein content of the cell is diminished does the ensuing specific activity value not dramatically increase? If one studies the sample mean values for the specific activities calculated, then one should observe that such an increase is evident in Phase I sclerota. However, there is an abrupt drop in the S. A. values on both accounts (plasmodia and Phase I) as the cell enters the fully sclerativated state of Phase II. The only exception to this is found in Phase II sclerotia of G-6-PO_4

and for all practical purposes, the S. A. value (instead of decreasing) remains the same as its previous value in the plasmodial state. So, we see the values taken from Table 7 increase from the plasmodial stage to Phase I sclerota, then decrease as they enter the fully sclerotized state of Phase II.

	Plasmodia	Phase I	Phase II
LDH	.3690	.5220	.1258
MDH	3.82	8.835	2.0429
G-6-PO_4	.1629	.3121	.1692

A number of plausible explanations could be offered as to why this occurs as it does. In the vegetative state one would expect the highest protein values to occur as the "free-living" plasmodia is manufacturing the necessary components in order to sustain this condition. Thus, one would expect a lot of free, unbound protein to be manifest throughout the cell entity. As the cell enters an early sclerotial state by emersion in a non-nutritive environment it should react to this abrupt transition from a comfortable, vegetative state. The cell may well alter its normal metabolic activities. Through a release or even a tying up of certain compounds brought about by this removal of nutrient materials, the specific

activities could be drastically altered. The enzymes used in this study are indicators of specific biochemical pathways within the cell entity. If there were a complete cessation of activity of one of the enzymes assayed, as the cell moved from a vegetative to a differentiated state, then such an observation would be very dramatic and informative. Unfortunately, this is not the case in these observations. However, there is an observable difference in the specific activity values obtained over time in this study, and it is this difference in values which must be accounted for and ascertained.

After twenty-four hours had elapsed, the total protein content (in trial No. 2) was found to be 228.51 mg. This figure represents a decrease of 7% in the total protein content over the plasmodial stage. Seven per cent may appear to be a small value but is actually quite dramatic when one recalls that this transposition took place in the relatively short span of only 24 hours.

From the Phase I stage the cell further differentiates into a definite sclerotium, the process requiring 96 hours of shaking in the starvation S media of Rusch. The enzymes per se may actually become more active as a result of the media change or they may just be rendered more readily available through metabolic readjustments which are taking place. Whatever the case, the trend or tendency is for the total protein content to be decreased while the

"activity" of the enzyme goes up in value.

In Phase II, one finds a completely different picture from that in Phase I sclerotia. The total protein content is still decreasing; however, the enzyme activity decreases as well. As far as the protein content is concerned, this can be plausibly explained as follows: As the cell enters a new phase or condition indicative of "self-preservation" a complete transition must take place in order for it to accomplish this end. Therefore, there is an obvious difference between the plasmodial state and the sclerotized state of this organism, both morphologically and biochemically.

Sullivan (57) investigated the biochemistry of dormancy in P. polycephalum and compared his analysis of sclerotia with active plasmodia. His findings were that the percentage of bound water to that of the active plasmodia was about one-half. More conducive to this report was that Sullivan regarded this difference in bound water content to major changes in carbohydrate metabolism since water-insoluble, long chain polysaccharides, glycogen and reducing sugars were present in decreased amounts in sclerotia. The decreased protein values across Table 7 could be reasoned out with the same train of thought. There are obvious morphological alterations taking place in conjunction with biochemical changes as well. The cell, in entering a state of sclerotization, could well be tearing

down certain protein structures, re-arranging them, and in the same instance inactivating other proteins and enzyme systems.

The amino acid analyses data lends supportive evidence to the fact that this could well be the case.

The correlation coefficients computed reveal, without doubt, no correlation whatsoever between any of the morphogenetic stages of the plasmodium and the differentiating sclerotium, as far as their enzyme activity values are concerned. This is not surprising since the organism is altering its biochemical pathways. The specific activities found for LDH plasmodium should not be the same for LDH sclerotium, as the organism is undergoing a complete transition in its biochemical integrity—a complete metamorphesis. Therefore, the negative correlation coefficients of the enzyme activities studied lend supportive evidence to the fact that the differentiating stages are divorced from one another, biochemically as well as morphogenetically.

Gel-electrophoresis on LDH, MDH, and G-6-PO_4 substantiated the fact that the enzyme system challenged was present and functional. There were no differences in the integrity or intensity of the stains (bands) over time, from one morphogenetic phase to another.

The reaction of ninhydrin with amino acids has led to the

development of several important methods for the estimation of amino acids and of reactions which liberate them. The colorimetric ninhydrin method used for this research is a very convenient and accurate means for estimations of amino acid content, when properly applied. Unlike other systems of detection (manometric and titrimetric) which depends on the presence of amino acids, a color reaction with ninhydrin is given by ammonia and other compounds with free amino groups. Therefore, one should not be surprised to find ninhydrin reactive compounds of an unidentifiable nature present on most all of the analyses performed.

Amino acids containing an α -amino group react with ninhydrin to give a colored derivitive, dipetohydrindylidenediketohydrindamine, plus the aldehyde of the amino acid and carbon dioxide. The colored product of the reaction has a characteristic absorption peak at 570 and the amount of color can be related to the quantity of amino acid present. With proline and hyroxyproline the reaction follows a different course and the product has its absorption maximum at 440mμ. The value of the color intensity of the aliquots read are converted to micromoles of the amino acid by comparison with the standard curve constructed for the particular amino acid being determined. This can readily be converted into percentages of each amino acid by dividing by the total micromoles.

Although the amino acids give different quantative responses to the color reagent, the construction of an accurate standard calibration curve should eliminate most error.

The methods of extraction used for the plasmodial phase, the Phase I sclerotium, the Phase II sclerotium and the spores was identical. Care was taken in the preparation of the extracts to allow no enzymatic activity to take place. As well, it is very unlikely that any non-enzymic hydrolysis would have caused breakage of the labile ester bonds of amino acids in aminoacyl-tRNA or aminoacylphospholipids or acylated polyol teichoic acids, found to be relatively important in maintaining a normal degree of integrity throughout the differentiating bacterial sporangium. Because of the care taken in preparation and protection of the samples for analyses, the data observed should provide an accurate estimation of the actual amino acid content of the particular phase being tested. Thus, any of the free amino acids found upon analysis should not be the result of release from covalent linkage, but should actually have been free within the entity tested.

The plasmodial phase possessed the highest value for total content of amino acids analyzed, 3.7817 μ Moles. As the differentiation process ensued, the total μ Mole content decreased with it, rendering values of 2.0622 μ Moles, 1.8874 μ Moles, and .6099 μ Moles, respectively for the Phase I and Phase II sclerotium, and

the spores. The trend, therefore, is that the amino acids are becoming less and less available as the organism enters and moves toward the fully differentiated state. Perhaps the amino acids are becoming bound to other compounds along the way, or perhaps they are under a new directive by the differentiating cell, which is no longer interested in synthesizing protein. As the cell prepares itself for the transition which is upcoming, other metabolic pathways become activated, such as those oriented towards the manufacturing of materials for the rigid, outer cell-wall component of the sclerotium and the spore. Since glutamic acid has been found to be a major component towards this end in bacterial spores, one might expect high levels of concentration of the material on the Phase II and sporangial graphs taken from the analyses. Instead, the percent composition of glutamic acid showed a steady decrease over time, quite divorced from the results expected. In the vegetative state the level of free glutamic acid was found to be 9.26%. In Phase I sclerotium, this figure dropped to 8.66%, followed by 3.78% for the Phase II sclerotium, with 1.77% reported for the spores. A plausible explanation would be that the level of free amino acids within the differentiating organism need not be the same as that of the vegetative cell. It is possible that glutamic acid, in the Phase II sclerotium and the spore, serves as a key intermediate in certain

metabolic reactions. When differentiation is complete, and metabolism ceases, glutamic acid might well be frozen in a byproduct state, and therefore missed in the specific analysis for its presence. For example, glutamate can form strong complexes with divalent cations, in the same manner as does DPA. Strong ternary complexes of DPA, Ca^{++}, and amino acids have been shown to exist in bacteria (46). It is possible that in the differentiated state, glutamic acid could well be involved in a network of such complexes, thereby masking out its true percentage. This argument might be applied to other amino acids which play a possible role in a DPA type complex, such as arginine.

Studying the data chart on the amino acid analyses of the various stages of differentiation in P. flavicomum, one detects several fluctuations which are outstanding. In the transition from the plasmodial stage to Phase II, proline dropped from 28% to 4%. As well, the serine-threonine complex increased from 17% to 35%. In the plasmodial stage to Phase II, one observes proline to drop a total of 20%, glycine increases from 1.97% to 10.8%, and alanine increases from 9% to 14%. Perhaps most dramatic is lysine, with an increase of 8% over its plasmodial stage reading as it reaches the sporangial stage. Histidine reveals a substantial increase over its plasmodial reading of .25% by attaining a value of 3.80% in the sporangial

phase. Arginine gives readings of .31% in the plasmodial stage and 2.83% for the spores.

Ammonia shows a substantial increase as the organism progresses toward the fully differentiated state. As ammonia is the by-product of metabolic and biochemical activity, this would indicate that something is certainly being broken down. Since protein was shown to decrease substantially in the enzyme activity analysis, one should suspect that this is probably the source of most of the by-product reaction. The differentiating cell is tearing down the vegetative protein moieties, and reassembling the amino acids into an arrangement more suitable to the differentiated state. Whatever role the amino acids finally assume, it is apparent that some mechanism exists for their direction and regulation into the differentiated state. From the data obtained and studied in this research on amino acid analyses, it seems evident that there are no specific, predictable trends for the increases or diminutions in the amino acid content of the various phases studied. The fully sclerotized state of the organism, as well as the sporangial state of the organism, does have a tendency to show very small pools of amino acid content when compared to the vegetative state and the early sclerotial phase of the organism.

Additional research in the area of enzyme analyses coupled

with amino acid data from the soluble pool of the organism could shed new light on the results obtained here. By using an extract from another phase, or harvest, one may well find substantial differences in the amino acid data and enzyme activities values over those obtained under the conditions applied in this research.

Most features of differentiation and morphogenesis in biological systems are poorly understood at the biochemical level. What initiates the differentiation process, and once initiated, what controls the orderly and sequential events which culminate in the morphogenetic product? These questions are constantly being asked about the complex, differentiating systems found in nature; however, continued study in the area of myxomycete research should allow a more detailed understanding of the biochemical and genetic processes involved.

SUMMARY

P. flavicomum variety 1 was found to sporulate best on a water substrate at pH 4.0, using a tungsten lamp source, in conjunction with an adequate starvation period of 96 hours.

Enzyme assays and disc-gel electrophoresis revealed the presence of the LDH, MDH, and glucose-6-phosphate enzyme systems. The specific activity compiled on these assays were statistically checked and subjected to computer analyses to depict an accurate picture, as to their overall significance. Specific activity values were found to fluctuate over time, as the organism entered into its differentiation cycle. Total protein values were found to decrease over time as the organism entered the morphogenetic cycle.

Amino acid analyses provided support for the enzyme assay data in the way of an explanation as to protein degradation as well as revealing an overall picture of the amino acid pool content of the organism over time.

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