ENZYME ACTIVITY CHANGES ASSOCIATED WITH DEVELOPMENT OF THE FRUITING MYXOBACTERIUM, MYXOCOCCUS XANTHUS.

by

ANEICY SYNE

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ABSTRACT

When an exponential culture of Myxococcus xanthus was induced with glycerol to form optically refractile myxospores, several changes in enzyme activities were observed. Isocitrate lyase, a key enzyme of the glyoxylate pathway, showed a 4-5-fold increase in activity after 2 hours of induction when all vegetative cells had converted to myxospores. A 4.7-fold increase in activity of uridine 5'-diphosphate-N-acetyl glucosamine pyrophosphorylase has also been reported (Filer et al., 1977b). This enzyme is required for uridine 5'-diphosphate-N-acetylgalactosamine formation, a major precursor of the myxospore coat. Alkaline phosphatase declined to a minimum 2 hours after induction and increased thereafter. Alkaline phosphatase activity may be related to germination of the myxospore and hence its low activity at the time of myxosporulation.

To determine whether these changes in enzyme activities were due to differentiation, and not a response to the presence of glycerol, the activities of the three enzymes were assayed in cells that were allowed to develop on solid medium. In this case, development is initiated by starvation. This process is radically different from glycerol-induced formation of myxospores, and is more closely related to the developmental response in nature. Under these conditions, isocitrate lyase activity was lowest 24 hours after onset of development, (the time of aggregation), and attained a maximum value at 72 hours when mature fruiting bodies containing refractile myxospores were formed. The activity began to increase at about 54 hours, the time when fruiting body myxospores became refractile. UDPG-pyro-

phosphorylase exhibited peak activities at 54 hours. Vegetative cells exhibited the greatest alkaline phosphatase activity. This value decreased throughout the developmental cycle.

Both isocitrate lyase and UDPG-pyrophosphorylase seem to function in biochemical pathways which lead to myxospore formation and myxospore coat formation. The behaviour of alkaline phosphatase correlates with its possible role in myxospore germination.

To my Mother and Father, without whose love and support, this would not have been possible.

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I am sincerely grateful to my supervisor, Dr. Peter Maeba, of the Department of Microbiology, University of Manitoba, for his help and encouragement throughout the course of this study. I would also like to thank my husband, Kazim, for his patience during the preparation of this thesis.

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A_{324}	absorbance at 324 nm
Δ_{324}	change in absorbance at 324 nm per minute
DNA	deoxyribonucleic acid
λ	lambda phage
mRNA	messenger RNA
RCF	relative centrifugal force
RNA	ribonucleic acid
TRIS	tris (hydroxymethyl) aminomethane
UDPG-pyrophosphorylase	uridine 5'-diphosphate-N-acetylglucosamine pyrophosphorylase
$Na_4P_4O_7$	sodium pyrophosphate

INTRODUCTION

INTRODUCTION

In an attempt to understand morphogenesis and developmental interactions in higher life forms, investigators have analysed biochemical events and correlated these to specific stages of morphogenesis to explain the complex processes involved in differentiation. Very few processes of differentiation are well understood or simple enough to allow a determination of the causes and controls involved. Analysis of differentiation in procaryotes offers a simpler system in which to elucidate the variables operating during morphogenesis with the reasonable hope that the findings can be extended to more complex systems.

The myxobacteria are gram negative, heterotrophic organisms which undertake a complex developmental pathway. They are readily cultivated in the laboratory and development proceeds in a manner that is very similar to that in the extensively studied eucaryotic slime mold, Dictyostelium discoideum. A study of enzyme levels in the latter revealed several enzymes show peak activities at specific stages of development. UDPG-pyrophosphorylase peaked at 21 hours after initiation of development, coinciding with the culmination of fruiting body formation (Roth and Ashworth, 1968). Alkaline phosphatase (a 5'-nucleotidase) activity attained a maximum, in vivo, during culmination and decreased to a minimum in mature fuiting bodies or sorocarps (Gezelius and Wright, 1965). The changes in enzyme activities have been linked to cellulose synthesis during the final stages of spore formation.

The presence of the glyoxylate cycle enzymes has been demonstrated in

E. coli (H.L. Kornberg, 1965), fungi (Olson, 1954; Saz, 1954) and in several species of bacteria. This anaplerotic pathway was only present when acetate or amino acids served as the sole carbon source. Two major enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase, reached peak activities in Myxococcus xanthus 2 hours after glycerol induction of sporulation (Orlowski et al., 1972). The peak in activities coincided with the time when myxosporulation is completed.

This investigation was undertaken to determine whether the levels of isocitrate lyase, alkaline phosphatase and UDP-N-acetylglucosamine pyrophosphorylase could be correlated to observable events in the development of M. xanthus.

HISTORY

HISTORY

Myxobacterales and Cytophagales comprise the two orders of the gliding bacteria according to Bergey's Manual of Determinative Bacteriology (1974). Except for the common property of gliding motility, they are otherwise unrelated. The Myxobacterales are bacteriolytic and unable to utilize carbohydrates as carbon and energy sources. The order Myxobacterales is further subdivided into four families on the basis of the morphology of the vegetative cells, myxospores and the fruiting bodies. The family Myxococcaceae is characterized by tapered rods and round or oval myxospores which are not enclosed in a sporangium. The family Archangiaceae differs from Myxococcaceae in that the myxospores are rod shaped. Cystobacteraceae differs from the above since the myxospores are enclosed in sporangia. The fourth family, Polyangiaceae, is characterized by rods with blunt ends and sporangia containing myxospores which look like the vegetative cells.

Detailed studies on the Myxobacterales have mostly been done with members of the Myxococcaceae family, particularly Myxococcus. In general myxobacteria are gram negative, procaryotic rods (3 - 10 μm in length and 0.5 - 1.0 μm in diameter) with cell walls characteristic of other gram negative bacteria. They are obligate aerobes with chemoorganotrophic metabolism and contain DNA with a guanosine plus cytosine content of 68 - 71 mole percent. They can hydrolyze other gram negative bacteria, proteins, fatty acid esters and some polysaccharides (Zusman, 1980). During vegetative growth, the cells glide over moist surfaces to promote spreading growth of cells.

The mechanism by which gliding motility occurs is not known but leads to swarming of cells on moist surfaces. It involves slow, rhythymic motion not affiliated with flagella. The movement of cells in groups is aided by the profuse production of slime that contains mannose, glucose, glucosamine and galactose (Sutherland and Thomson, 1975). It functions both in holding the swarm together and in proteolysis. Gnosspelius (1978) showed that the slime actually acts to denature proteins thus facilitating the degradation of microorganisms and complex substrates. A genetic analysis conducted by Hodgkin and Kaiser (1979) showed that gliding motility was controlled by at least 31 different gene products which functioned in two independent gene systems. Gene system A controlled the movement of single cells whilst gene system S was responsible for single and group movement of cells. Linkage analysis studies with several mutants revealed that gene system A involved at least 22 different loci and gene system S, at least 9 different loci. Kaiser (1979) further showed that cells possessing a complete gene system S were piliated. Thus system S may control the production of pili which could be involved in gliding or cellular interactions.

Myxobacteria are unique among procaryotes in that they exhibit a complex developmental cycle, similar to eucaryotic cellular slime molds. When nutrient supply is depleted on solid medium, vegetative cells aggregate to form fruiting bodies, within which the cells differentiate into myxospores. When conditions permit, the myxospores germinate into vegetative cells which grow and divide by binary transverse fission. Throughout the cycle of development, cell interactions and co-operative behaviour is demonstrated.

The most thoroughly studied species is Myxococcus xanthus. The fruiting body of this species consists of a simple mound of cells in contrast to the elaborate, multilobed, treelike structures of others. The myxospores represent a resting state which is resistant to heat, dessication and other physical and chemical agents.

Several factors that stimulate aggregation and the initiation of fruiting body formation have been found. For instance, the developmental cycle can be induced by limitation of any one of the amino acids, lysine, threonine or diaminopimelic acid, or by starvation for inorganic phosphate (Rosenberg et al., 1973). It was suggested that these amino acids repress aspartokinase activity resulting in starvation for diaminopimelic acid and blockage of cell wall growth which leads to fruiting. If isoleucine, leucine and methionine were present however, aspartokinase activity was stimulated and the fruiting process was inhibited. Bretscher and Kaiser (1978) have formulated a minimal medium, A1, in which the amino acids phenylalanine, isoleucine, leucine, valine and methionine were essential for continuous growth of M. xanthus. If any of these essential amino acids were omitted or limited in the medium, the fruiting process was initiated.

Yajko and Zusman (1978) have shown that cyclic AMP and ADP may also be involved in the fruiting process. Cyclic AMP levels were shown to double in M. xanthus during aggregation on starvation medium. Also added cyclic AMP and ADP were found to stimulate fruiting body formation on solid medium which could support vegetative growth but not fruiting due to nutrient limitation (Campos and Zusman, 1975). The exact mechanism

leading to fruiting body formation is obviously under multiple controls that remain to be elucidated. Present knowledge indicates that the level of specific metabolites and cell number play a critical role. Approximately 5.0×10^8 cells/mL were necessary for fruiting body formation to occur (Wireman and Dworkin, 1975).

The cellular interactions involved during aggregation may be facilitated by a myxobacterial hemagglutinin (Cumsky and Zusman, 1979). The myxobacterial hemagglutinin was not present in vegetative cells but appeared in cells plated on a fruiting medium after 6 to 8 hours of development. The specific activity of the lectin was shown to peak between 18 and 24 hours of development, the time when aggregation was completed (Stern, 1979; Cumsky and Zusman, 1979). Hemagglutinin activity was not inhibited by simple sugars but it was inhibited by fetuin, a fetal calf serum glycoprotein, suggesting that it binds to complex receptors containing a peptide sequence with carbohydrate residues. The fate of the lectin is not known with any certainty but it may be excreted outside the cell and then degraded by proteases later on in the developmental cycle. Myxobacterial hemagglutinin represents an excellent developmental marker and probably plays an important role in cell interactions during aggregation by binding to cell surface receptors.

The result of aggregation is a mound of cells coated with a layer of hardened slime (immature fruiting body). If starvation conditions persist, the vegetative cells within the fruiting body morphologically convert to round or oval, resistant myxospores. Wireman and Dworkin (1979) showed that only one fifth of the population of cells present at 24 hours of

development differentiated into myxospores at 72 hours. The remaining four fifths of the population autolysed. It was speculated that the lysis of cells was necessary for provision of nutrients to the survivors. They suggested that the developing cells had 3 possible fates. Cells could be classified as vegetative, intermediate or competent. Competent cells differentiate into myxospores whilst intermediate cells had 2 alternate possibilities: autolysis or conversion to competent cells. Whatever the reason for autolysis by 80% of the population, it is certain that it must in some way relate to the survival of the species.

It is possible to by-pass the series of events leading to fruiting body myxosporulation by the addition of glycerol, 0.5 M final concentration, or other compounds with primary or secondary alcohol groups, to an exponentially growing liquid culture (Sadler and Dworkin, 1966). After addition of glycerol, vegetative cells formed into myxospores synchronously within 2 hours. Glycerol spores and fruiting body myxospores were similar with the exception of a surface coat protein, designated protein S, in the fruiting body spores (Inouye et al., 1979). The wall of glycerol spores is composed of 75% polysaccharide, 14% protein, 8% glycine and less than 1% organic phosphorus. The polysaccharide was mainly galactosamine and glucose (50%) with some neutral sugars (25%) (Kottel et al., 1975). Disaggregation of the peptidoglycan of vegetative cells by trypsin revealed that it had a patch-like, discontinuous structure separated by non-peptidoglycan material (White et al., 1968). The resulting flexibility may facilitate gliding and morphogenesis of a rod to a spherical myxospore. In contrast, the peptidoglycan of the myxospore

was a continuous layer and resistant to several chemical treatments suggesting that the polysaccharides were covalently bound to each other.

During glycerol induction, the synthesis of a galactosamine derivative and glycine dramatically increased during spore coat formation. This coincided with a rapid increase in UDP-N-acetylglucosamine pyrophosphorylase activity, 1 and 2 hours after glycerol induction (Filer et al., 1977b). This enzyme participates in the synthesis of UDP-N-acetylgalactosamine, a major component of the myxospore coat. Also, during this period the activity of the glyoxylate cycle enzymes increased (White et al., 1968). This increased activity of the glyoxylate cycle enzymes could provide precursor carbon for gluconeogenesis as well as glyoxylate for glycine synthesis. The presence of amino acid transaminases and dehydrogenases in M. xanthus that can utilize glyoxylate as a substrate in vitro further substantiates this possibility (White et al., 1968). The resistance of myxospores to proteases, glycosidases, dessication and sonication may be attributed to the addition of galactosamine derivative and glycine to the peptidoglycan and to the increased cross-linking of the peptidoglycan. It is interesting that the time when galactosamine was accumulating in the coat polymer coincides with the acquisition of resistance to sonication (White et al., 1968).

The mechanism by which glycerol induces myxospore formation is not known. It is clear that fruiting body myxosporulation and glycerol-induced sporulation represent 2 separate genetic events since most glycerol non-inducible mutants can form myxospores in fruiting bodies (Parish et al., 1976). Two hypotheses to explain the mechanism of glycerol induction have

been proposed (Zusman, 1980). The first involves unregulated transcription and was postulated because of the similarity of the agents which induce myxospore formation in M. xanthus and activate the galactose of E. coli and lambda phage operons. Glycerol, ethylene glycol and 1,3-propanediol all induce myxosporulation in Myxococcus. These same compounds promoted increased expression of the galactose operon in E. coli even if active galactose repressor was present (Nakanishi et al., 1974). They also partially induced prophage lambda and caused expression of promotor-defective λ operons (Nakanishi et al., 1974). If unregulated transcription is the mechanism by which solvents induce myxospores, the process must differ significantly from the ordered events of spore formation that accompanies fruiting body development.

In E. coli it has been shown that detachment of the bacterial chromosome from the cell membrane blocked the initiation of chromosome replication so that different promotor sites became available for transcription (Lark, 1969). The second hypothesis proposes that glycerol may cause a disturbance in the cell membrane which triggers chromosome detachment from the cell membrane, making available promotor sites of sporulation genes (Zusman, 1980). These 2 hypotheses remain to be tested.

Metabolic studies on M. xanthus are essential if the variables operating during development are to be defined. Inouye et al., (1979) investigated the pattern of protein synthesis during development on fruiting medium and glycerol induction. They classified the synthesis of the major proteins into 4 types: accumulation proteins, peak proteins, late proteins and constant proteins. The rate of synthesis of protein S,

an accumulation protein, increased throughout the developmental cycle to a maximum of 15% of total protein synthesis. Protein S was absent in glycerol-induced spores suggesting it may function in the aggregation of spores in fruiting bodies. The rate of synthesis of proteins T, M, N, P, O and R peaked midway in development between 20 and 25 hours and these were classified as peak proteins. Late proteins were only synthesized at a very late period of development. For example, protein U was not synthesized until 35 hours after onset of development and its rate of synthesis was highest at 45 hours. Protein Q, a constant protein, was synthesized at a constant rate throughout development. These changes in protein synthesis coincided with different morphological changes that accompany fruiting body formation.

Changes in nucleic acid biosynthesis during differentiation have been extensively studied. Kimchi and Rosenberg (1976) showed that, during glycerol induction, chromosome replication was not initiated, but on-going chromosome replication was completed. They further showed that completion of chromosome replication was not required for spore formation since vegetative cells whose DNA synthesis had been inhibited by nalidixic acid still formed glycerol spores. However nalidixic acid prevented the first cell division following germination. Furthermore messenger RNA made early in development contained sequences not found in vegetative cell messenger RNA (Okano *et al.*, 1970). This new messenger RNA may be required for the synthesis of enzymes needed for the conversion of the vegetative rods to myxospores and subsequent germination.

Biochemical differentiation, as defined by Killick and Wright (1974), is a specialization process during which a cell (or its progeny) accumulates significant amounts of materials not present at an earlier point in time. The fluctuations in enzyme activities can be used to obtain information of the various biochemical changes associated with specific stages of development. At least 40 enzymes showed changes in specific activity during differentiation in the cellular slime mold, Dictyostelium discoideum and their role in development could, in part, be elucidated (Killick and Wright, 1974). Because of the similarity of morphogenesis in Dictyostelium discoideum and Myxococcus xanthus, it seemed reasonable to assume that an understanding of the biochemical changes in M. xanthus would help to explain the developmental events occurring in the bacterium.

Watson and Dworkin (1968) demonstrated the presence of glycolytic, gluconeogenic and Kreb's cycle enzyme activities in crude extracts of vegetative cells and myxospores of M. xanthus. Also both cell types lacked the enzymes hexokinase and pyruvate kinase. Since M. xanthus was unable to use glucose, glycogen or starch as a carbon source, it was postulated that glycolysis and gluconeogenesis served solely to metabolize endogenous carbohydrate. Amino acids could also enter the Kreb's cycle via acetate and thus serve as carbon and energy sources. Except for isocitrate lyase, the glycolytic and Kreb's cycle enzymes did not show any differences in activity in vegetative cells or myxospores. Bland et al., (1970) showed that both major enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase, were synthesized de novo

during glycerol-induced morphogenesis. After 1 hour of glycerol induction both enzyme activities rapidly increased, and after 2 hours, started declining to levels found in vegetative cells. Orlowski et al., (1972) extended these studies and found that the decline in activities coincided with the time of conversion to refractile (by phase optics) spheres. The changes in enzyme activities were not due to glycerol metabolism per se since cells incubated in a medium with ethylene glycol as an inducer showed the same enzyme changes. Also, a mutant which cannot myxosporulate when induced with glycerol or ethylene glycol did not show the characteristic changes in glyoxylate cycle enzymes. During glycerol induction, respiration decreased by 80% and cellular neutral polysaccharide synthesis increased by 20% (Bacon et al., 1975) coinciding with the increase in glyoxylate cycle enzymes. This possibly represents the shift of carbon flow from oxidative respiration to polysaccharide synthesis via the glyoxylate shunt. These findings led to the conclusion that the glyoxylate cycle played an essential role in the differentiation process of glycerol-induced cells of M. xanthus.

The glyoxylate cycle is a modified form of the tricarboxylic acid cycle in that it bypasses two oxidative steps found in the TCA cycle. This anaplerotic pathway is required for replenishment of intermediates of the Kreb's cycle which are continuously being removed for biosynthesis of cell components. The glyoxylate cycle also enables M. xanthus to use fatty acids or acetate for carbohydrate biosynthesis.

The cause for the decrease in the glyoxylate cycle enzymes after glycerol-induced myxosporulation is complete is still uncertain. Orlowski

and White, (1974) demonstrated increased intracellular proteolytic activity in developing myxospores and suggested that this could be in part responsible for the decrease in glyoxylate cycle enzyme activity. It is worth noting that at the time isocitrate lyase activity is decreasing, net protein synthesis is also declining. Therefore the decrease in enzyme activity was not due to a dilution of the enzyme by continued synthesis of other protein (Orlowski et al., 1972). Isocitrate lyase activity may be controlled by excretion of the enzyme from the cell (Orlowski et al., 1974). For example, the enzyme UDP-galactose polysaccharide transferase of D. discoideum was excreted from cells by an energy-requiring process during fruiting body formation (Sussman and Osborn, 1964). However Orlowski and White (1974) were not able to detect isocitrate lyase activity in the culture medium of glycerol-induced cells. The information available on inactivation of isocitrate lyase suggests that inactivation requires continuous metabolic energy and protein synthesis during the first 50 - 120 minutes of induction. The exact mechanism is not known.

Filer et al., (1973) showed the presence of aspartokinase in M. xanthus which proved to be an essential marker of development. Threonine and lysine, both derived from aspartate, repressed aspartokinase activity and stimulated fruiting body formation. Also isoleucine and methionine stimulated aspartokinase activity and inhibited the fruiting process. It was suggested that a decrease in aspartokinase activity inhibited lysine biosynthesis resulting in starvation for diaminopimelic acid and blockage of vegetative cell wall growth which led to induction of the developmental cycle. This hypothesis is supported by the fact that aspartokinase activity rapidly decreased during the first 2 hours of glycerol induction, the time when the glyoxylate cycle enzymes showed peak

activities and myxosporulation was near completion (Rosenberg et al., 1973).

M. xanthus is capable of utilizing amino acids as carbon and energy sources because of the presence of amino acid transaminases and dehydrogenases (Kottel et al., 1974). Vegetative cells and 4 hour old glycerol-induced myxospores contained glutamate, alanine and glycine dehydrogenases as well as glutamate-glyoxylate diamino-transferase and alanine-glyoxylate aminotransferase. It is possible that the glyoxylate formed via isocitrate lyase is converted to capsular glycine of the myxospore coat by the glycine dehydrogenase and/or transaminases described by Kottel et al., (1974).

Five enzymes involved in the synthesis of UDP-N-acetylgalactosamine from fructose-1,6-diphosphate increased 4.5 - 7.5-fold in activity after 2 hours of glycerol induction. This was not surprising since over two-thirds of the myxospore coat is polysaccharide, N-acetylgalactosamine being the main component (Filer et al., 1977a). These enzymes, glutamine synthase, glucosamine-6-phosphate acetyltransferase, UDP-N-acetyl-glucosamine pyrophosphorylase, UDP-N-acetylglucosamine-4-epimerase, fructose 1,6-diphosphate phosphatase and L-glutamine-fructose, 6-phosphate transamidase, all showed peak specific activities between 1 and 2 hours of glycerol induction (Filer et al., 1977b). Analysis of the composition of the purified myxospore coat showed that it consisted mainly of N-acetylgalactosamine (50%) with 25% glucose, 14% protein and 7-8% glycine (White et al., 1968). Consequently, the synthesis of the coat polymer could be followed by the incorporation of [^{14}C]-glycine and [^{14}C]-acetate into the acid-insoluble polymer. [^{14}C]-acetate was recovered in the

acetyl portion of UDP-N-acetylgalactosamine and N-acetylglucosamine-6-phosphate (Filer et al., 1977a). The studies showed that the rate of incorporation of label into myxospore coats were correlated with the increased enzyme activities observed in sporulating cells (Filer et al., 1977a). Since these changes occurred during the formation of the polysaccharide coat of myxospores, they are excellent markers of glycerol-induced myxosporulation.

The ultimate fate of myxospores is germination, nutritional conditions permitting. The biochemical events leading to germination is still under speculation. Ramsey and Dworkin (1968) showed that, if the spore density were sufficiently high, they could germinate in distilled water. This process involved the excretion of a substance called the germination factor. The requirement for a high cell density for germination was interpreted as a need for a high concentration of this germination factor. Since myxospores suspended in water excreted orthophosphate, it was suggested that excreted orthophosphate was the germination factor (Dworkin, 1973). Dworkin reported that alkaline phosphatase activity in glycerol-induced cultures of M. xanthus increased dramatically 3-4 hours after glycerol induction and remained at a high level for the first 2 hours of germination. It therefore seemed possible that the alkaline phosphatase was responsible for the generation of orthophosphate. On the other hand, Voelz et al., (1966) have demonstrated the presence of polyphosphate granules in the cytoplasm of M. xanthus grown in casitone-magnesium medium and these granules may be the source of orthophosphate.

Alkaline phosphatase plays a critical role in the development of the cellular slime mold Dictyostelium discoideum. The enzyme activity increased about 6-fold during the differentiation process but decreased to a minimum in mature fruiting bodies or sorocarps (Gezelius et al., 1965). The investigators showed that the high intracellular phosphate concentration in mature spores inhibited the enzyme activity by 93% in vitro. They suggested that end-product inhibition by phosphate was important when the organism differentiated under extreme conditions of starvation.

Most of the biochemical markers of development have been identified in glycerol-induced cultures of M. xanthus. Because conclusions cannot be drawn by only considering the behaviour of these enzymes during induced morphogenesis, this work was undertaken to determine whether the activities of isocitrate lyase, UDP-N-acetylglucosamine pyrophosphorylase and alkaline phosphatase could serve as markers during colonial morphogenesis.

MATERIALS AND METHODS

MATERIALS AND METHODS

Materials

All reagents were obtained from Sigma Chemical Company with the following exceptions: $MgCl_2 \cdot 6H_2O$ was obtained from J.T. Baker Chemical Company, Philipsburg, New Jersey; $Na_4P_4O_7$ (anhydrous) was obtained from The British Drug Houses Limited; $MgSO_4$ was obtained from Fisher Scientific Company.

Buffers

Tris - 500 mM Tris HCl; pH 7.5

Tris - 500 mM Tris HCl; pH 8.0

Tris acetate - 0.1 M Tris, pH 7.8; 0.1 M acetate containing 0.001 M mercaptoethanol.

Methods

Organism

Myxococcus xanthus PM200 was grown in 1% (w/v) Difco casitone containing 0.08 M $MgSO_4$ in a G-76 Gyrotory water bath shaker (New Brunswick Scientific Company) at 28°C. Stock cultures were routinely made by adding 50% sterile glycerol (v/v) to a final concentration of 0.5 M to log phase cultures. Two millilitres of culture were aliquoted to sterile vials which were stored at -70°C until needed.

Fruiting Body Formation

The following procedure describes how cells were induced to form fruiting bodies in a more or less synchronous fashion. Stock cultures were inoculated into 40 mL of 1% Difco casitone medium containing 0.08 M $MgSO_4$ and grown to a density of 8 x 10^8 cells/mL (200 Klett units, red filter). Ten millilitres of this culture was transferred to 400 mL of 1% Difco casitone medium containing 0.08 M $MgSO_4$ in 2-litre Erlenmeyer flasks and shaken at 150 rpm in a Model G-53 Gyrotory Shaker (New Brunswich Scientific Company) at 28°C. At mid-log phase (about 200 Klett units), the cells were harvested in a RC-5 Sorvall Superspeed refrigerated centrifuge (Sorvall Incorporated) with a Sorvall type GS-3 rotor at 9000 RCF for 20 minutes. The cell pellets were washed in 500 mM Tris-HCl buffer, pH 7.5 and suspended in the same buffer to a density of 3 x 10^{10} cells/mL. Ten to 20 microliter amounts were spotted on starvation medium with the aid of a peristaltic pump. After the spots had soaked into the medium, the plates were incubated at 30°C. Plates were removed at 12, 24, 30, 36, 48, 54, 60 and 72 hours. The 0 hour sample was harvested as soon as the spots were dry. The cells were harvested by scraping the agar surface with a razor blade. Pellets were stored at -70°C overnight. Starvation medium was composed of 0.8% Difco agar (Difco Laboratories) and 0.2% agarose in distilled water. This was autoclaved for 20 minutes and cooled before 0.08 M $MgSO_4$ was added. The medium was distributed into petri plates (150 mM diameter), allowed to solidify and refrigerated until needed.

Glycerol Induction

Myxospore formation in liquid culture was achieved by the method of Wireman and Dworkin (1975). Mid-log phase cultures were made 0.5 M with sterile glycerol and shaken continuously in a G-53 Gyrotory shaker. Samples were removed at 0, 1, 2, 3 and 4 hours, cooled on ice and centrifuged at 9000 RCF for 20 minutes. The 0 hour sample was cooled on ice before glycerol addition. Synchronous conversion to myxospores occurred within 2 hours. Cell pellets were washed with 500 mM Tris-HCl buffer, pH 7.5 or pH 8.0 for the isocitrate lyase or alkaline phosphatase assay, respectively and stored at -70^{o}C overnight.

Preparation of Cell Extracts

To prepare cell extracts from glycerol-induced cultures, cell pellets were suspended in 3 mL or 500 mM Tris-HCl buffer, Ph 7.5 or pH 8.0 for the isocitrate lyase or alkaline phosphatase assay, respectively. The cell suspension was placed in bottles with 10 times its weight of acid washed glass beads (75-150 microns) and broken using a Bronwill homogenizer, Model 2876 (Bronwill Scientific, Rochester, N.Y.). Vegetative cells were broken by 4-30 second exposures and myxospores were broken by 8-30 second exposures. The suspension was then centrifuged at 3000 RCF for 2-3 minutes to remove glass beads. The supernatant was centrifuged at 12,000 RCF for 5 minutes to remove cell debris. The resultant clear supernatant was used as the crude cell extract for the enzyme assays.

Samples from petri plates were suspended in 3 mL of 500 mM Tris-

HCl buffer, pH 7.8 or pH 8.0 (for the assay of isocitrate lyase or alkaline phosphatase) or 0.1 M Tris-acetate buffer, pH 7.8, for the assay of UDPG pyrophosphorylase. The cell suspensions were subjected to sonication using an Ultrasonic Dismembrator, Model 300 (Fisher Scientific Company) fitted with a microtip. Vegetative cells were subjected to 5-30 second exposures and myxospores were subjected to 10-30 second exposures. The cell suspension was centrifuged at 12,000 RCF for 10 minutes. The supernatant fluid was used as the crude enzyme preparation.

All preparations were done at 0-4^{o}C.

Enzyme Assays

Isocitrate lyase activity was assayed by the method of Maloy et al. (1980), a modification of the procedure of Dixon and Kornberg (1959). Enzyme was mixed with a freshly prepared reaction mixture containing 6 µmole $MgCl_2$, 4 µmole phenylhydrazine hydrochloride, 12 µmole cysteine HCl, and Tris buffer, 500 mM, pH 7.8 in a total volume of 1.0 mL. The formation of glyoxylic acid phenylhydrazone was measured spectrophotometrically in the presence of 8 µmole trisodium isocitrate as the increase in the absorbance at 324 nm using a Gilford spectrophotometer. One unit of enzyme activity is the amount of enzyme catalyzing the formation of 1.0 µmole glyoxylic acid phenylhydrazone in one minute and a ΔA_{324} of 0.100 per minute is equivalent to 0.0087 units of enzyme (Dixon and Kornberg, 1959). Specific activity was expressed as µmole glyoxylic acid phenylhydrazone formed per minute per milligram of protein.

Alkaline phosphatase activity was measured by the method of Malamy and Horecker (1964). Cell extract, 0.05 M p-nitrophenylphosphate and 0.5 M Tris-HCl buffer, pH 8.0 were mixed in a total volume of 1.0 mL. The formation of p-nitrophenol was measured spectrophotometrically as the decrease in absorbance at 420 nm using a Gilford spectrophotometer. To convert ΔA_{420} into μmoles of p-nitrophenol formed, a standard curve of A_{420} against various concentrations of p-nitrophenol was constructed. The p-nitrophenol was directly proportional to enzyme activity since the p-nitrophenylphosphate substrate for alkaline phosphatase is cleaved to give p-nitrophenol. A ΔA_{420} of 1.0 per minute is equivalent to 1.37 μmoles p-nitrophenol per mL. Specific activity was expressed as μmoles of p-nitrophenol formed per minute per mg protein.

UDPG-pyrophosphorylase activity was measured by the method of Hanson et al. (1966). The formation of glucose-1-phosphate was followed by coupling the pyrophosphorolysis of UDP-glucose with NADP reduction. NADPH formation was measured spectrophotometrically as an increase in the absorbance at 340 nm. The assay mixture consisted of 0.02 mL of the following reagents:

Mg acetate 0.1 M, in 0.1 M Tris-acetate buffer, pH 7.8

$Na_4P_2O_7$, 0.1 M, in 0.1 M Tris-acetate buffer, pH 7.8

NADP, 0.02 M

UDP-glucose, 0.02 M

Solution: 50 units of phosphoglucomutase and 2.5 units of glucose-6-phosphate dehydrogenase per mL. Varying amounts of enzyme (0.05-0.2 mL)

were added and the total volume adjusted to 1.0 mL with Tris-acetate buffer, 0.1 M, pH 7.8. Specific activity was expressed as μmoles of UDP-N-acetylglucosamine formed per minute per mg protein - where an increase in absorbance at 340 nm of 0.63 corresponds to 0.1 μmole of UDP-N-acetylglucosamine (Horecker and Kornberg, 1948).

Protein Determination

Protein was determined by the Bio-Rad Protein Assay Method (Bio-Rad Laboratories) using bovine albumin as a standard. The principle of the method is based on the differential colour change of coomassie brilliant blue G-250 when it binds to protein. The absorbance maximum of an acidic solution of coomassie brilliant blue G-250 shifts from 465 nM to 595 nM when bound to protein.

RESULTS

RESULTS

Preliminary Studies

The aim of this project was to determine if 3 specific enzymes could serve as biochemical markers of development in Myxococcus xanthus.

Bland et al., (1970) previously reported that both major enzymes of the glyoxylate pathway, isocitrate lyase and malate synthase, were synthesized de novo in M. xanthus during glycerol induction. Orlowski et al., (1972) noted that stationary-phase cells did not show the characteristic increase in activity of the glyoxylate cycle enzymes. As a preliminary study it was necessary to obtain cells during the exponential phase of growth. Figure 1 shows the growth curve of M. xanthus PM200. Exponential-phase cells, 180-200 Klett units (red filter) were used for all experiments.

Glycerol Induction of Myxospore Formation

The sequence of events occurring during the morphological conversion of a rod to a sphere after glycerol addition is shown in Fig. 2. There was a decrease in turbidity upon the addition of glycerol to the culture. Such changes in turbidity have been reported when solutes were added to cultures of a number of gram-negative bacteria (Mager et al., 1956). The decrease in optical density does not indicate a decrease in cell density. According to the graph, there was no visible morphological change for the first 35 minutes but by 40 minutes the rods began to shorten. Most of

Figure 1. Growth curve of Myxococcus xanthus PM200.

The culture was grown in 40 mL of 1% Difco casitone medium containing 0.08 M $MgSO_4$ and incubated in a Gyrotory water bath shaker (New Brunswick Scientific Company) at $32^{o}C$. At the time intervals indicated, cell density was measured using a Klett-Summerson colorimeter with a red filter.

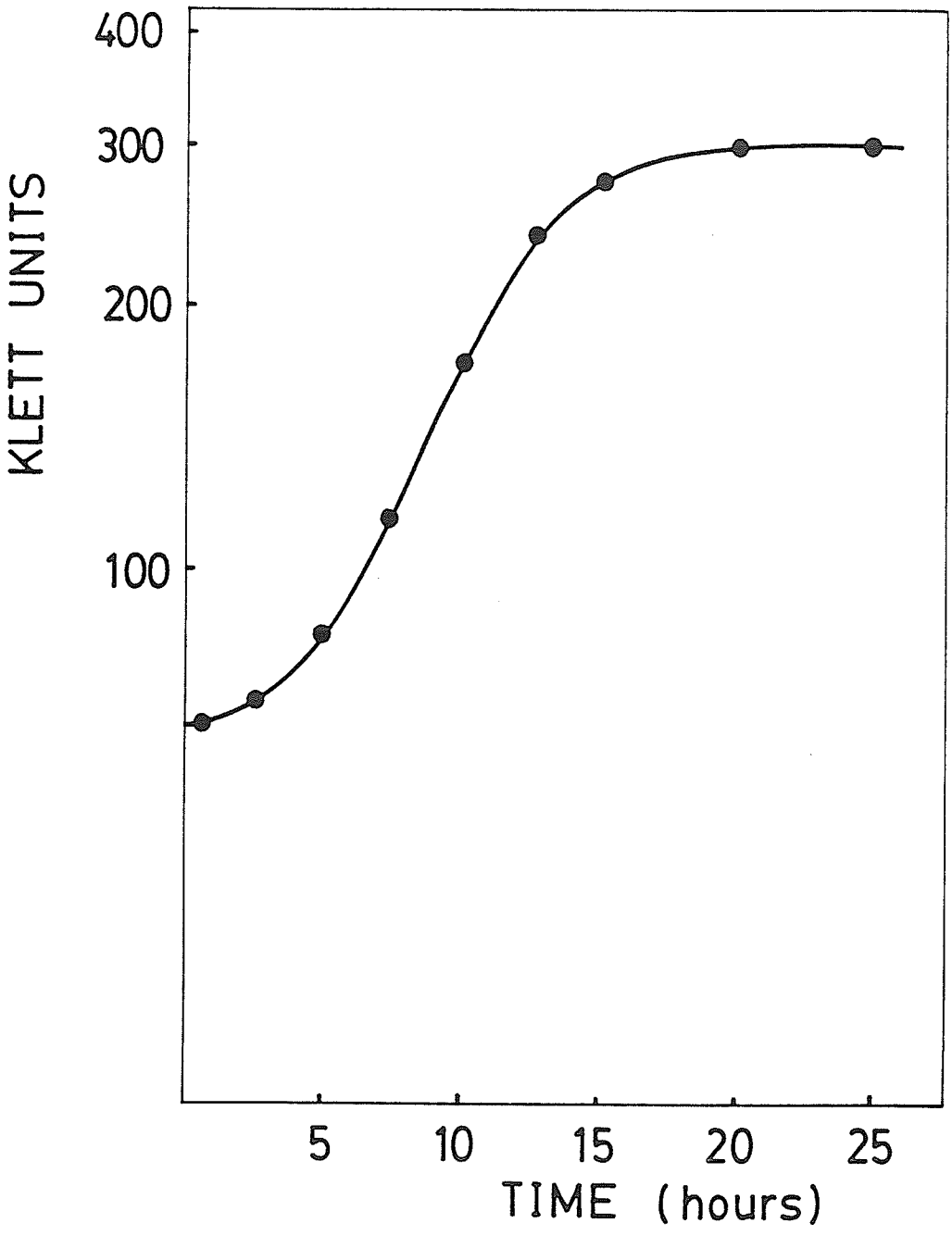
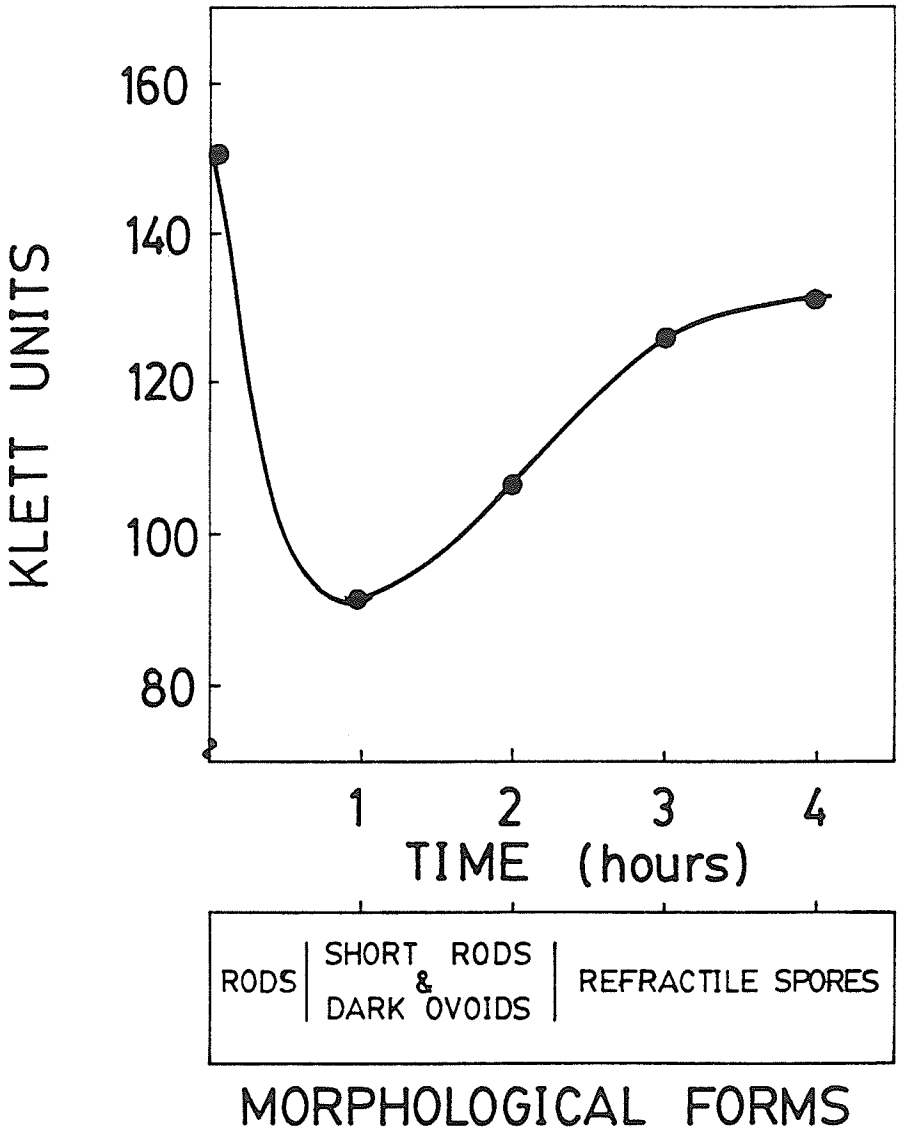

400
300
200
100
KLETT UNITS
5
10
15
20
25
TIME (hours)

Figure 2. Glycerol induction of myxosporulation.

The culture was induced with 0.5 M sterile glycerol as described in Methods. Cell density was measured in a Klett-Summerson colorimeter and samples taken at various times were viewed under a phase-contrast microscope. The time at which the different morphological forms appeared is given along the abscissa.


160
140
120
100
80
KLETT UNITS
1
2
3
4
TIME (hours)
RODS
SHORT RODS & DARK OVOIDS
REFRACTILE SPORES
MORPHOLOGICAL FORMS

the cells converted to dark ovoids between 80-100 minutes. The entire conversion to refractile spores occurred 2 hours after glycerol induction after which there were no further physical changes but as the spores became refractile, the optical density gradually increased. These results were identical to that obtained by Dworkin and Gibson (1964).

Isocitrate Lyase Activity in a Glycerol-Induced Culture

Isocitrate lyase was assayed in extracts of cells taken at specific times after glycerol induction as described in Methods. The linearity of reaction rate was assessed with varying amounts of enzyme. Extracts from induced and vegetative cells were used so that a wide range of activity could be measured. The results, (Fig. 3), show the reaction rate was linear, but began to level off at higher enzyme activities. In the assays that follow, the enzyme concentration was adjusted so that the activity was in the linear range and did not exceed a rate of 0.16 ΔA_{324}/minute.

Figure 4 shows the specific activity of isocitrate lyase in extracts of cells harvested at specific times of glycerol induction. The activity began to increase from a basal level of 0.14 μmoles of product/min/mg protein, 1 hour after glycerol addition and peaked after 2 hours at 0.68 μmoles of product/min/mg protein. The increase in activity occurred after cells had shortened into oval forms during the period when the property of refractility was acquired (Fig. 2). There was a 5-fold increase in isocitrate lyase activity at 2 hours as compared to the level found in vegetative cells. After 2 hours of induction the enzyme activity rapidly

Figure 3. Velocity of the isocitrate lyase reaction.

Enzyme extract was assayed as described in Methods. The extracts were prepared from vegetative cells, (•—•), and cells harvested 2 hours after glycerol induction, (○—○).

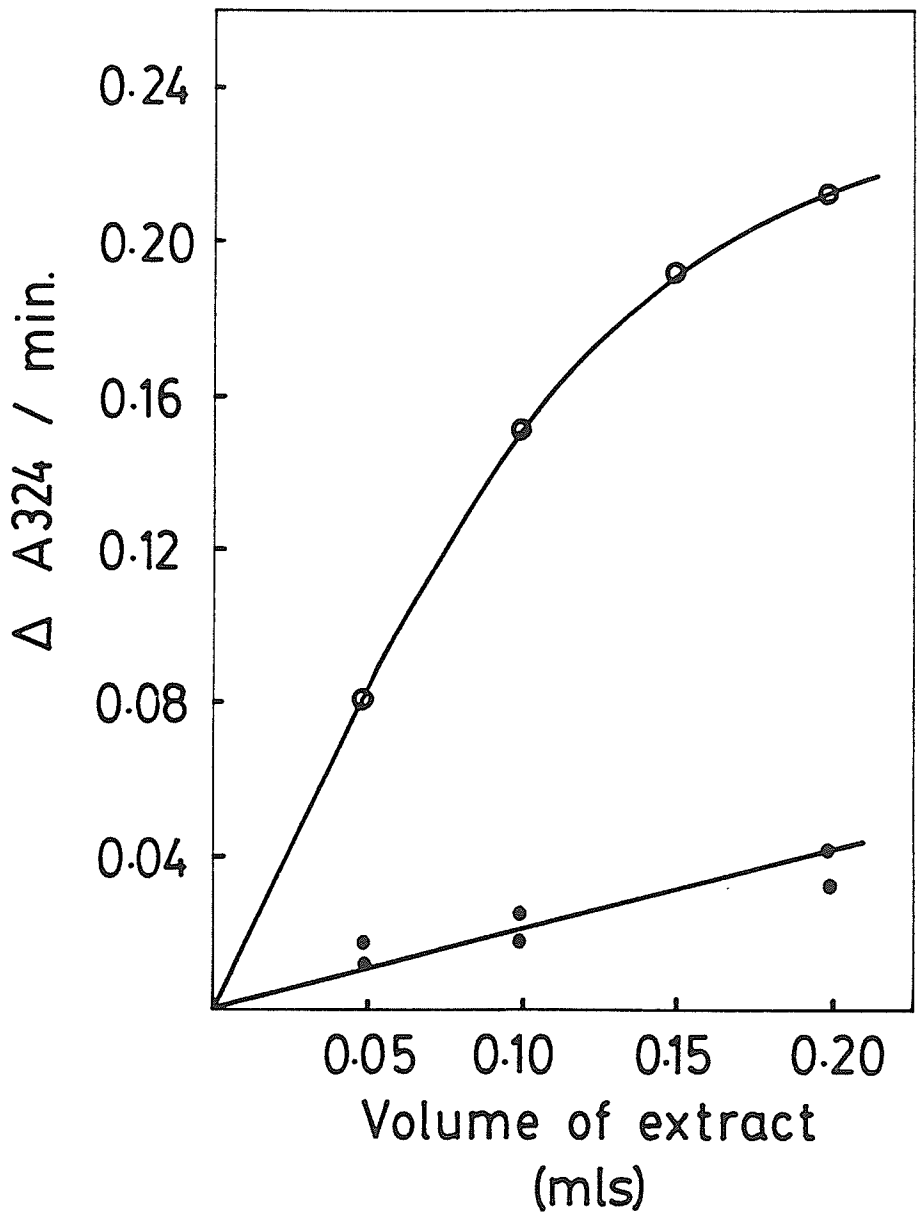
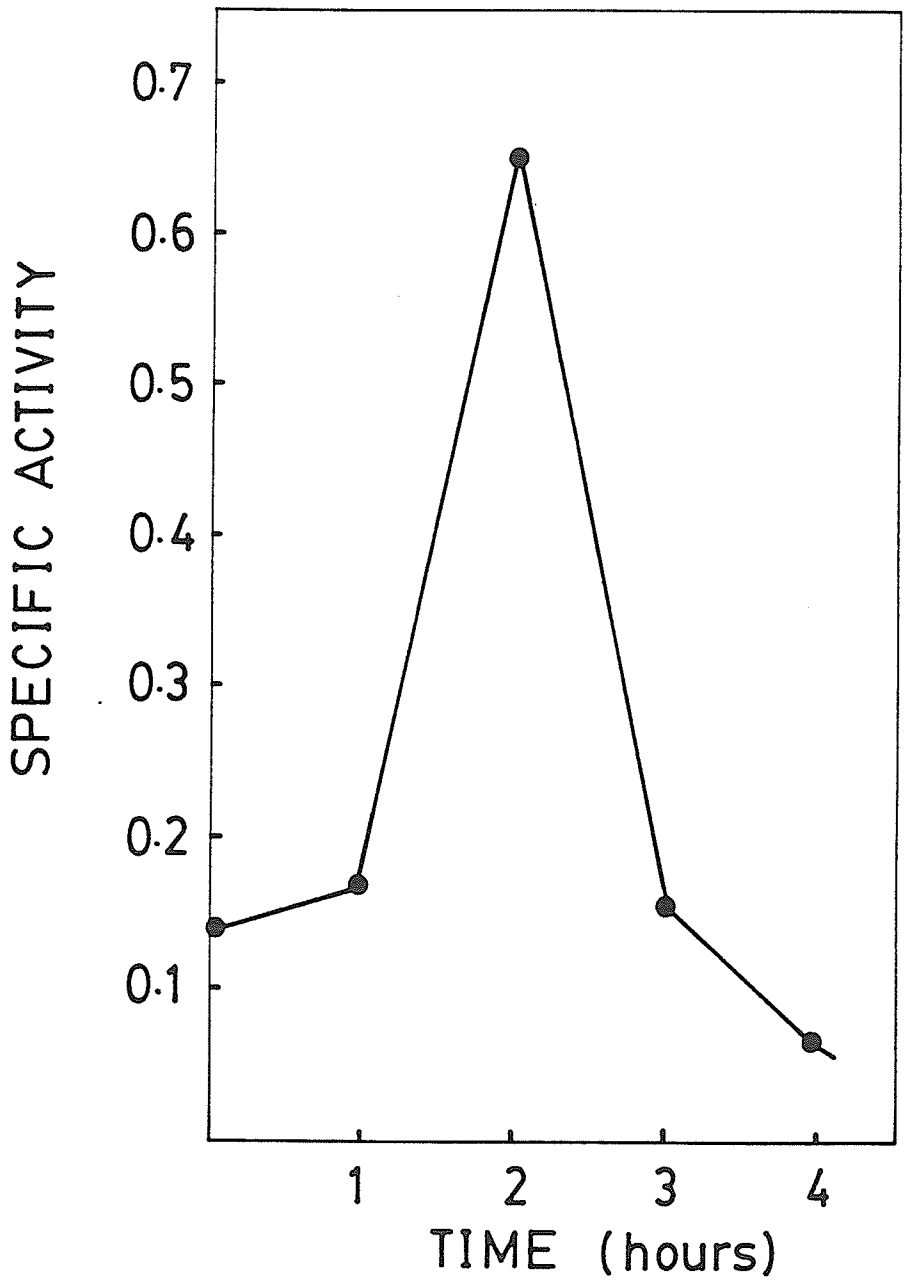

0·24
0·20
0·16
0·12
0·08
0·04
Δ A324 / min.
0·05
0·10
0·15
0·20
Volume of extract
(mls)

Figure 4. Isocitrate lyase activity in a glycerol induced culture.

The culture was induced with glycerol and isocitrate lyase activity was measured as described in Methods. Specific activity is expressed as μmoles glyoxylate phenyl hydrazone formed per minute per milligram of protein in the cell extract.


SPECIFIC ACTIVITY
0·7
0·6
0·5
0·4
0·3
0·2
0·1
1
2
3
4
TIME (hours)

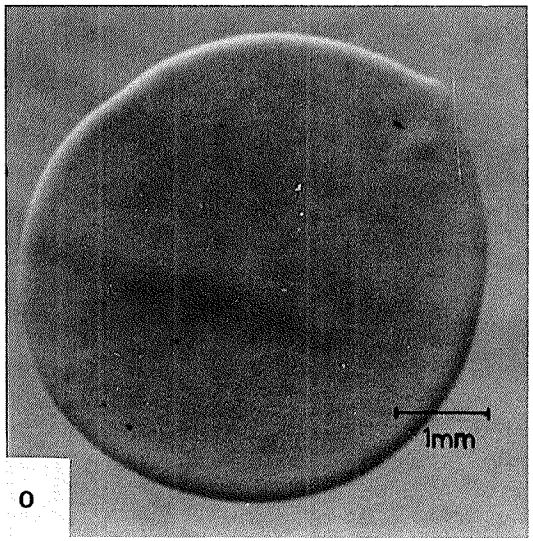
decreased so that by 3 hours, the level was that found in vegetative cells. This confirms previous work that showed similar changes in activity of isocitrate lyase (History) and indicates that the glyoxylate cycle was induced during glycerol-induced cellular morphogenesis of M. xanthus.

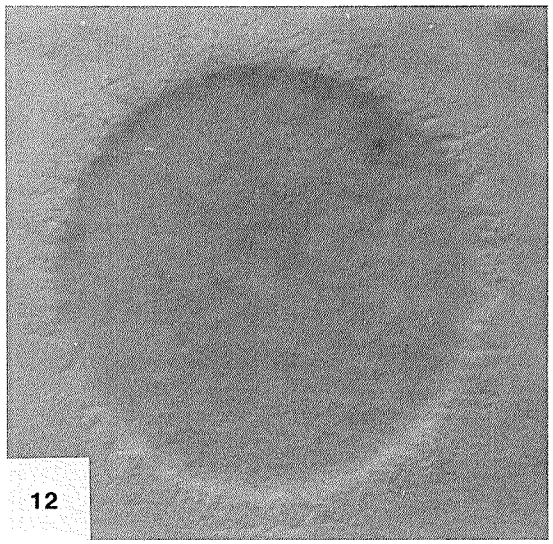
Isocitrate Lyase Activity During Fruiting Body Formation

The activity of isocitrate lyase was also assayed in cells engaged in fruiting body formation. Ten to 20 μL of concentrated cells (3×10^{10} cells/mL) were spotted on buffered agar and allowed to develop at 30°C as described in Methods. Figure 5 shows the time course of development of such cells. After the spot had soaked into the agar, that is, 0 time, the surface and edges were smooth and homogeneous. After 12 hours of incubation the surface was wrinkled and fringes were observed at the edges indicating aggregation of cells. Aggregation continued until 30 hours. By 36 hours raised structures were visible representing immature fruiting bodies. Some of the cells within the fruiting body at this time were oval in shape. At 48 hours the fruiting bodies were more pronounced and darker in colour. Most of the cells within the fruiting body had converted to dark, oval cells by this time. Refractility, as observed by phase contrast microscopy, was not acquired until 54 hours of incubation, although by this time, there were no further changes in the external appearance of the fruiting body. It was necessary to assay the enzyme activity beyond 48 hours as myxosporulation was not completed until about 72 hours of development.

Figure 5. Differentiation of M. xanthus PM200.

Cells were plated on fruiting medium as described in Methods. Pictures were taken of the developing cells under a dissecting microscope. The numbers represent the hours of incubation at $30^{\circ}C$. The bar in the 0 hour picture gives the scale. Each spot originates from 3-6 x 10^8 vegetative cells.


1mm
0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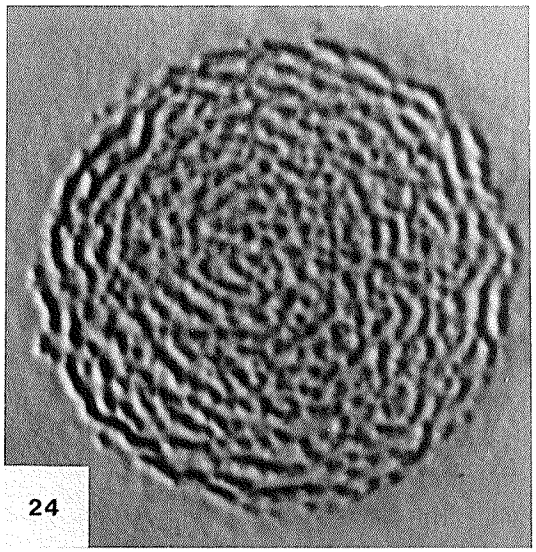
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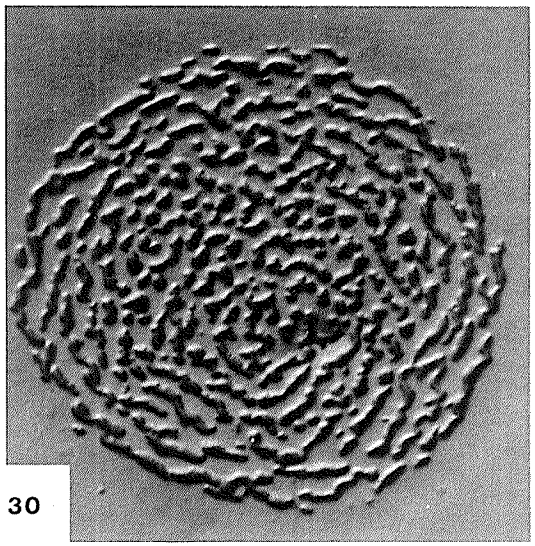
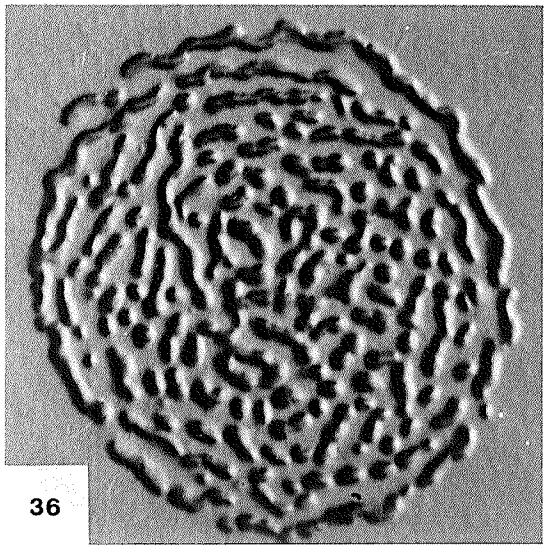
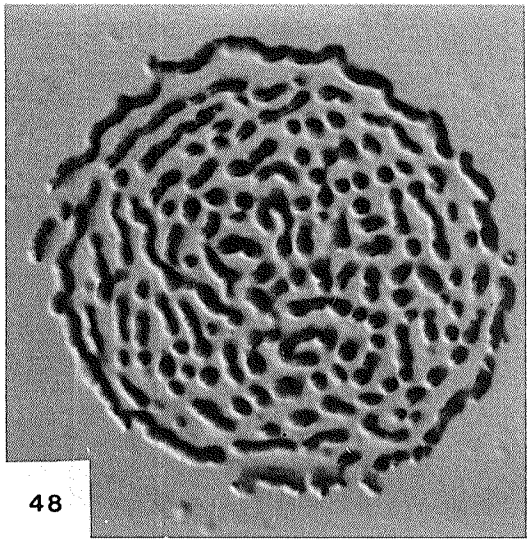

30


36


48

The linearity of the reaction catalyzed by isocitrate lyase extracted from developing cells is shown in Fig. 6. All assays were adjusted to fall in the linear region. Cells were scraped from plates at different times and extracted as described in Methods. Enzyme activity and protein concentration were measured and the calculated specific activities are shown in Fig. 7. There was approximately a 5-fold increase in enzyme activity in the 72 hour old myxospores as compared to vegetative cells. The enzyme activity remained at the level found in vegetative cells throughout aggregation (12 to 30 hours). The increase in enzyme activity occurred in 2 stages. A gradual increase was observed between 36 and 48 hours, when immature fruiting bodies were formed and cells had become oval. A second rapid increase in activity occurred between 48 and 72 hours (Fig. 7) when myxospores became refractile. The rapid disappearance of isocitrate lyase activity observed after glycerol-induced myxosporulation was completed (Fig. 4) did not occur after fruiting body spores were formed. This may be due to the asynchronous, slow conversion of rods to spores within the fruiting bodies. In both glycerol induced and fruiting cultures, maximum isocitrate lyase activities coincided with the conversion of ovoid cells to refractile myxospores. This observation suggests that the glyoxylate cycle was operative in the terminal stages of spores formation.

Alkaline Phosphatase Activity During Glycerol Induction

Alkaline phosphatase was assayed in glycerol induced cultures at specific time intervals as described in Methods. The linearity of the

Figure 6. Velocity of the isocitrate lyase reaction.

Enzyme extracts were prepared as described in Methods. The extracts were prepared from vegetative cells, (⊶⊸), and from cells 60 hours into development, (●⊸●).

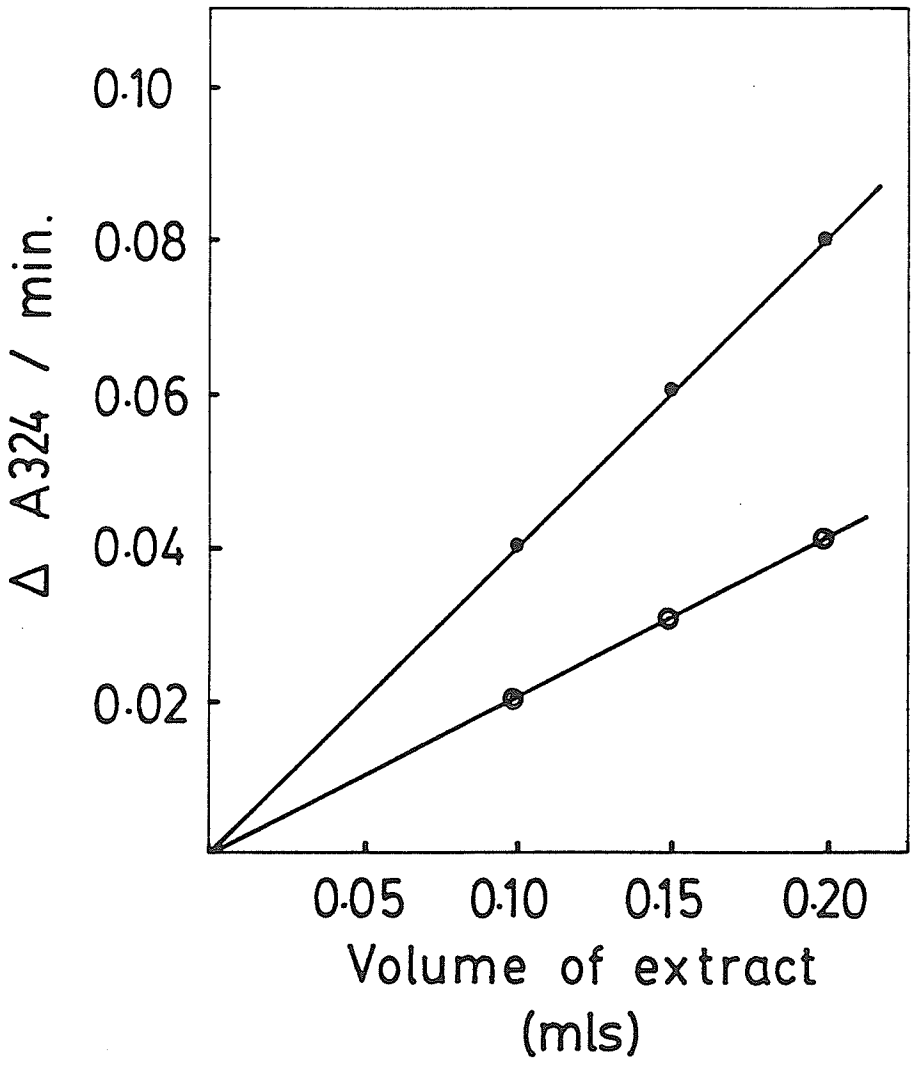
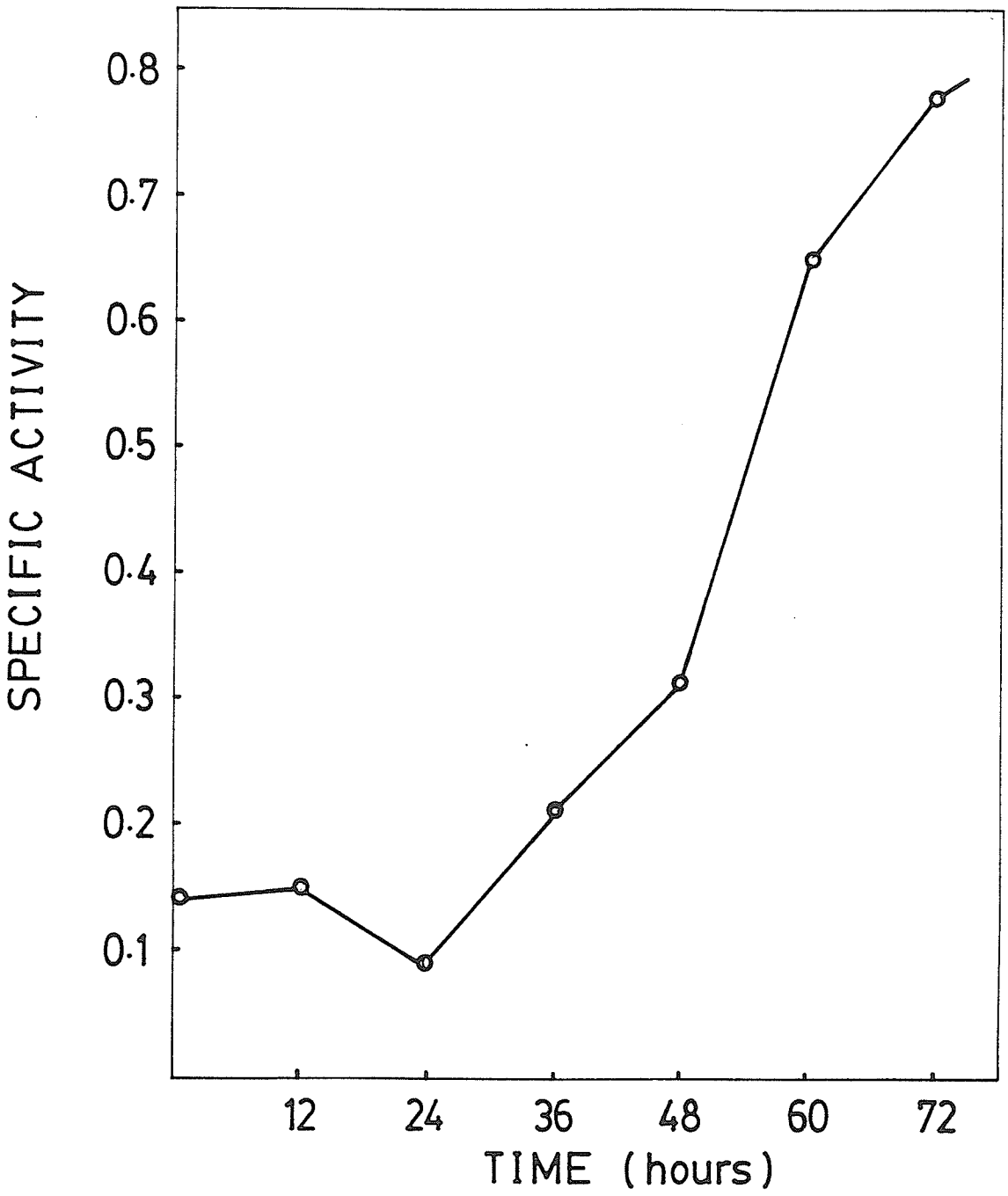

0·10
0·08
0·06
0·04
0·02
Δ A324 / min.
0·05
0·10
0·15
0·20
Volume of extract
(mls)

Figure 7. Specific activity of isocitrate lyase during fruiting body formation.

Cells were harvested and extracted at the indicated times and enzyme activity and protein were assayed as described in Methods. Specific activity is expressed as μmoles glyoxylate phenylhydrazone formed per minute per milligram of protein.


SPECIFIC ACTIVITY
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
12
24
36
48
60
72
TIME (hours)

reaction catalyzed by alkaline phosphatase is shown in Fig. 8. All assays were adjusted to fall in the linear region. The specific activity of alkaline phosphatase at different times during glycerol induction is shown in Fig. 9. The specific activity remained more or less constant for 2-3 hours after induction. At this time myxosporulation was completed. Thereafter, a rapid rise in activity occurred between 3 and 4 hours. The 4 hour sample had a 2-fold greater activity than the vegetative cells. These results indicate that glycerol induced myxospores were metabolically active 4 hours after induction and that increased alkaline phosphatase was not required for spore formation.

Alkaline Phosphatase Activity During Fruiting Body Formation

Alkaline phosphatase was assayed at specific stages during the fruiting process as described in Methods. Enzyme activity and protein concentration were measured and the calculated specific activities are shown in Fig. 10. The enzyme activity rapidly decreased throughout development and no measurable amount of activity was present after 48 hours of incubation. These results indicate that alkaline phosphatase was not required for spore formation in fruiting bodies.

UDPG Pyrophosphorylase Activity During Fruiting Body Formation

UDPG pyrophosphorylase is involved in myxospore coat polymer formation in glycerol-induced cultures of M. xanthus (Filer et al., 1977b). They reported a 4.7-fold increase in pyrophosphorylase activity at 2 hours of

Figure 8. Velocity of the alkaline phosphatase reaction.

Enzyme extract was assayed as described in Methods. The extracts were prepared from vegetative cells, (●—●), and cells harvested 4 hours after glycerol induction, (○—○).

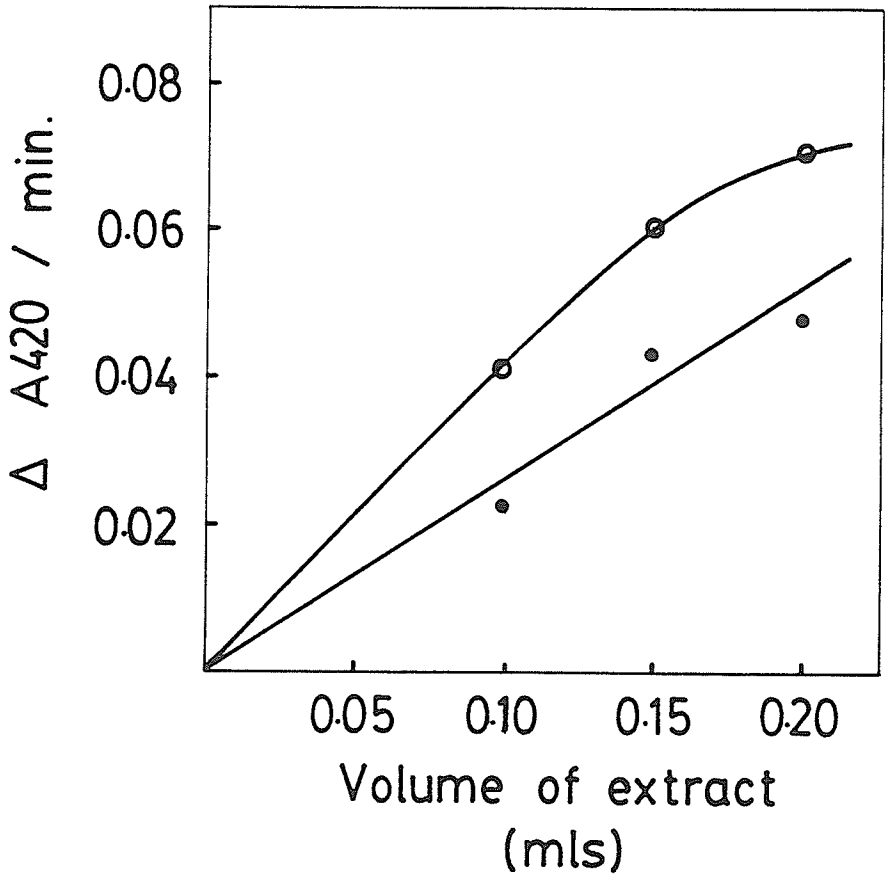

0·08
0·06
0·04
0·02
Δ A420 / min.
0·05
0·10
0·15
0·20
Volume of extract
(mls)

Figure 9. Specific activity of alkaline phosphatase during glycerol induction.

The culture was induced with glycerol and enzyme activity and protein were assayed as described in Methods. Specific activity is expressed as μmoles p-nitrophenol formed per minute per mg protein.

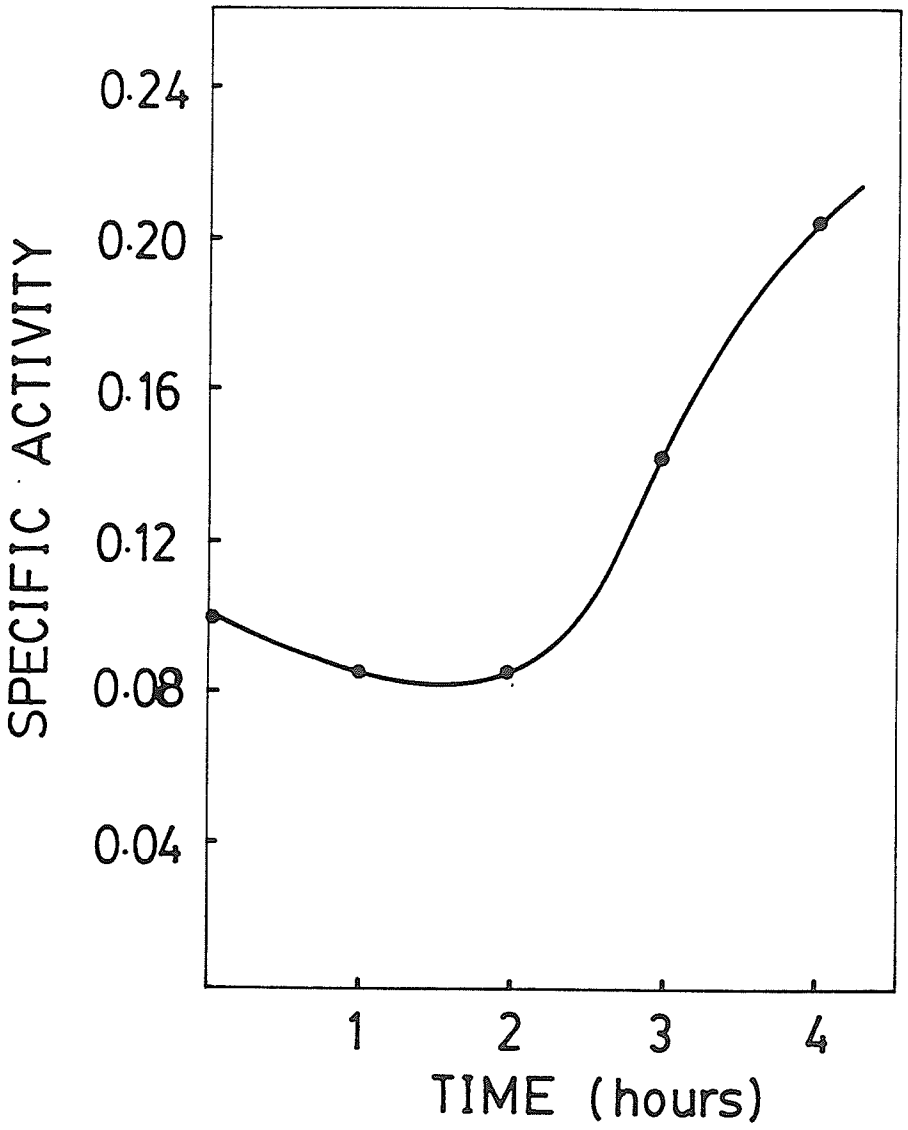
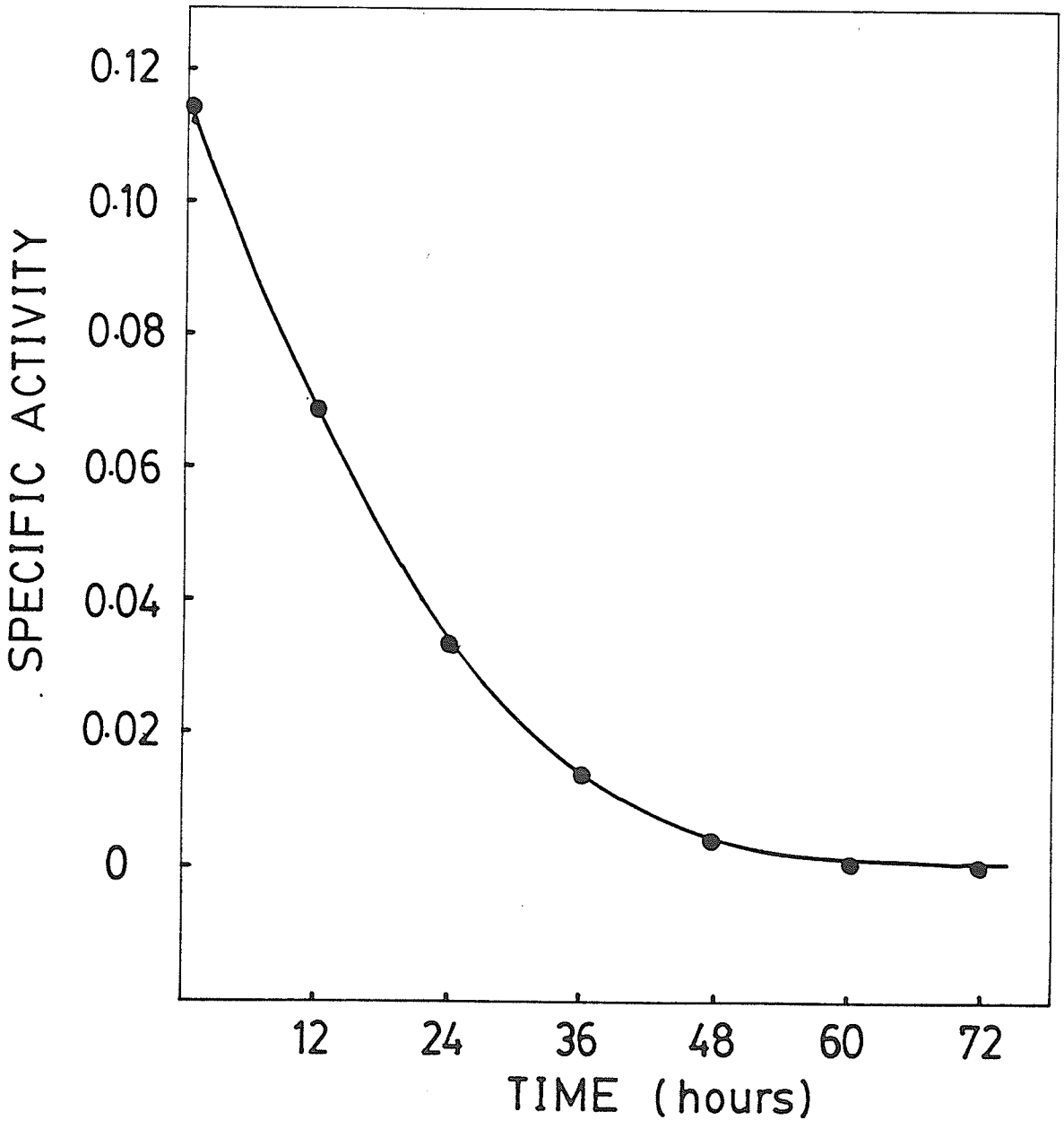

SPECIFIC ACTIVITY
0·24
0·20
0·16
0·12
0·08
0·04
1
2
3
4
TIME (hours)

Figure 10. Specific activity of alkaline phosphatase during fruiting body formation.

Cells were harvested and extracted at the indicated times. Enzyme activity and protein were assayed as described in Methods. Specific activity is expressed as μmoles p-nitrophenol formed per minute per mg protein.


SPECIFIC ACTIVITY
0·12
0·10
0·08
0·06
0·04
0·02
0
12
24
36
48
60
72
TIME (hours)

glycerol induction, the time when myxosporulation was completed. The enzyme activity was therefore assayed in cells undergoing fruiting body formation to determine whether a correlation existed between the time of enzyme induction in the 2 types of cultures.

The linearity of the reaction catalyzed by UDPG pyrophosphorylase extracted from developing cells is shown in Fig. 11. All assays were adjusted to fall within the linear range. Cells were scraped from plates at specific times and extracted as described in Methods. Enzyme activity and protein concentration were measured and the calculated specific activities are shown in Fig. 12. The specific activity remained close to the level found in vegetative cells for the first 24 hours of development and then gradually increased for an additional 24 hours. There was a burst in activity at 54 hours, the time when most of the myxospores within the fruiting bodies acquired refractility. The activity at 54 hours was 2-3-fold greater than that found in the vegetative cells and remained at a very high level until spore formation was completed at 72 hours. Filer at al., (1977b), reported an induced specific activity of 0.1 μmoles of product formed/min/mg protein in 2 hour old glycerol-induced myxospores. These results show an induced specific activity of 0.11 μmoles of product formed/min/mg protein in 54 hour old fruiting body myxospores. The data here, in addition to that of Filer et al. (1977b) indicate that maximum pyrophosphorylase activity was associated with the conversion of ovoid cells to refractile myxospores suggesting that the enzyme is operative in the terminal stages of myxospore formation.

The above results are summarized in Figs. 13 and 14. During fruiting

Figure 11. Velocity of the UDPG pyrophosphorylase reaction.

Enzyme extract was assayed as described in Methods. The extracts were prepared from vegetative cells, (⟜⟞), and from cells 60 hours into development, (⟜⟞).

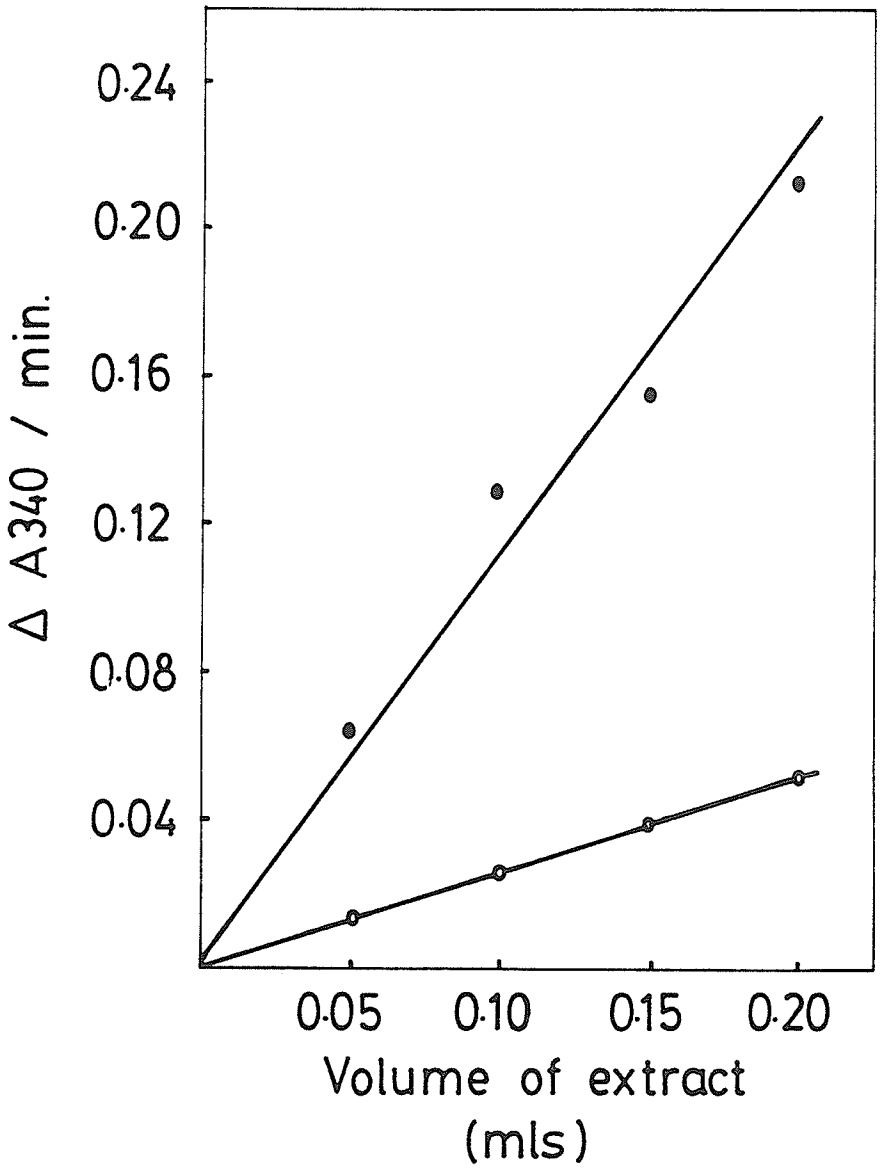
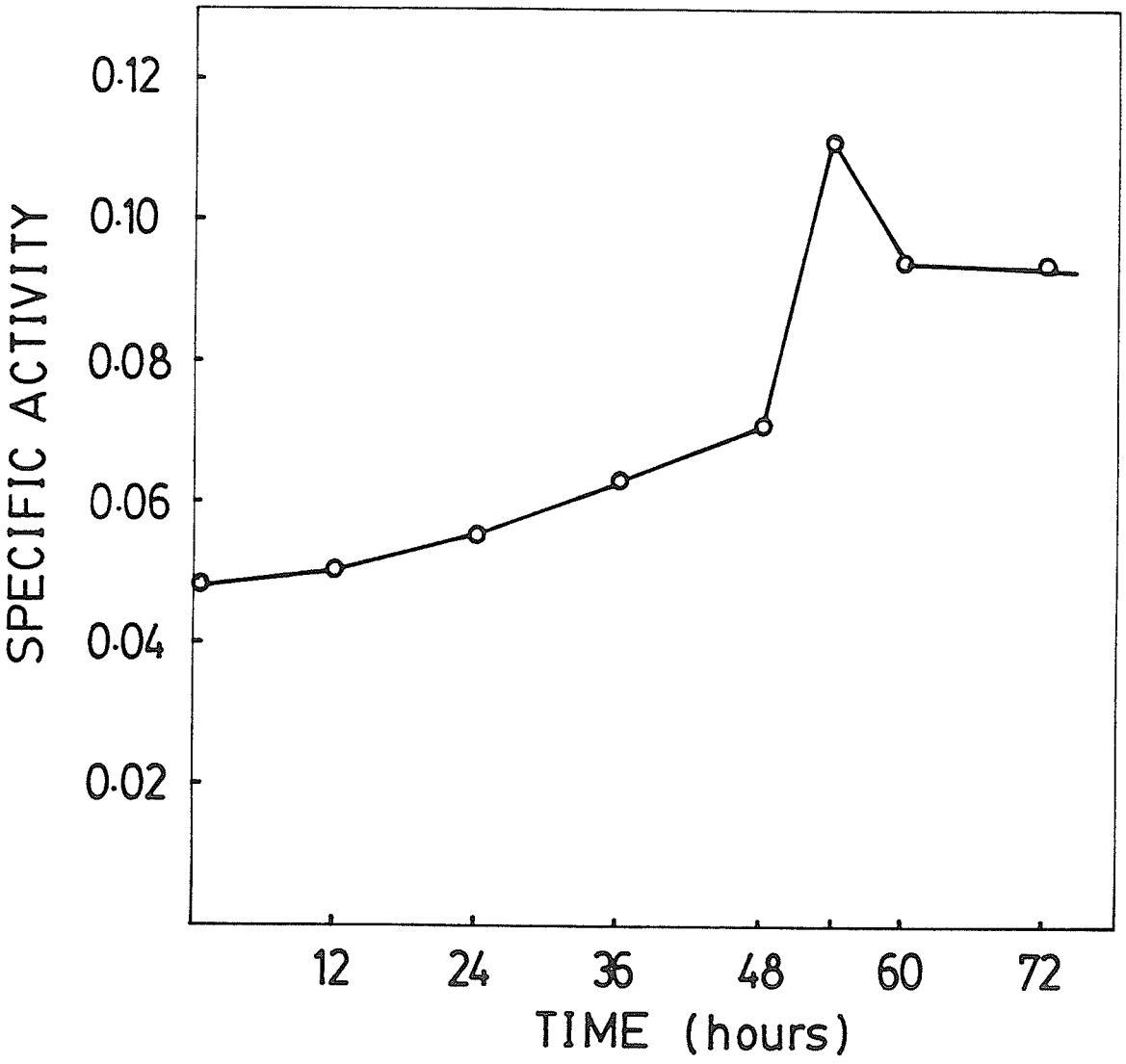

0·24
0·20
0·16
0·12
0·08
0·04
Δ A340 / min.
0·05
0·10
0·15
0·20
Volume of extract
(mls)

Figure 12. Specific activity of UDPG pyrophosphorylase during fruiting body formation.

Cells were harvested and extracted at the indicated times. Enzyme activity and protein were assayed as described in Methods. Specific activity is expressed as μmoles UDP-N-acetylglucosamine produced per minute per mg protein.


SPECIFIC ACTIVITY
0·12
0·10
0·08
0·06
0·04
0·02
12
24
36
48
60
72
TIME (hours)

body formation (Fig. 13) and glycerol induction (Fig. 14), isocitrate lyase and UDPG pyrophosphorylase showed peak specific activities at the terminal stages of spore formation. At 2 hours of glycerol induction at least 90% of the ovoid cells had converted to refractile myxospores, coinciding with the increased enzyme activities. During fruiting body formation some of the myxospores acquired refractility by 54 hours of development and this continued until 72 hours, coinciding with the increased enzyme activities. These results suggest that isocitrate lyase and UDPG pyrophosphorylase are operative during the final stages of myxospore formation when dark, ovoid spores become refractile. Alkaline phosphatase activity during glycerol induction was lowest during myxosporulation suggesting that the enzyme is not required for myxospore formation. During fruiting body formation, the peak activity was found in the vegetative cells at 0 hour time. Other markers of growth during fruiting body development are shown in Fig. 13. Myxobacterial hemagglutinin accumulates between 18 and 24 hours coincident with aggregation (Stern, 1979; Cumsky and Zusman, 1979). Protein S, an accumulation protein, accumulates during aggregation and its rate of synthesis is maintained at a high level throughout development (History).

Figure 13. Kinetics of the 3 enzymes during fruiting body formation.

Specific activities are expressed as percentages of the peak specific activity of each corresponding enzyme. The peak specific activities were 0.78 μmoles glyoxylate phenylhydrazone formed/min/mg protein for isocitrate lyase, (●—●), 0.112 μmoles p-nitrophenol formed/min/mg protein for alkaline phosphatase, (×—×), and 0.11 μmoles UDP-N-acetylglucosamine formed/min/mg protein for UDPG pyrophosphorylase, (○—○). The time of accumulation of myxobacterial hemagglutin (MBHA) and protein S is given along the abscissa (see text).

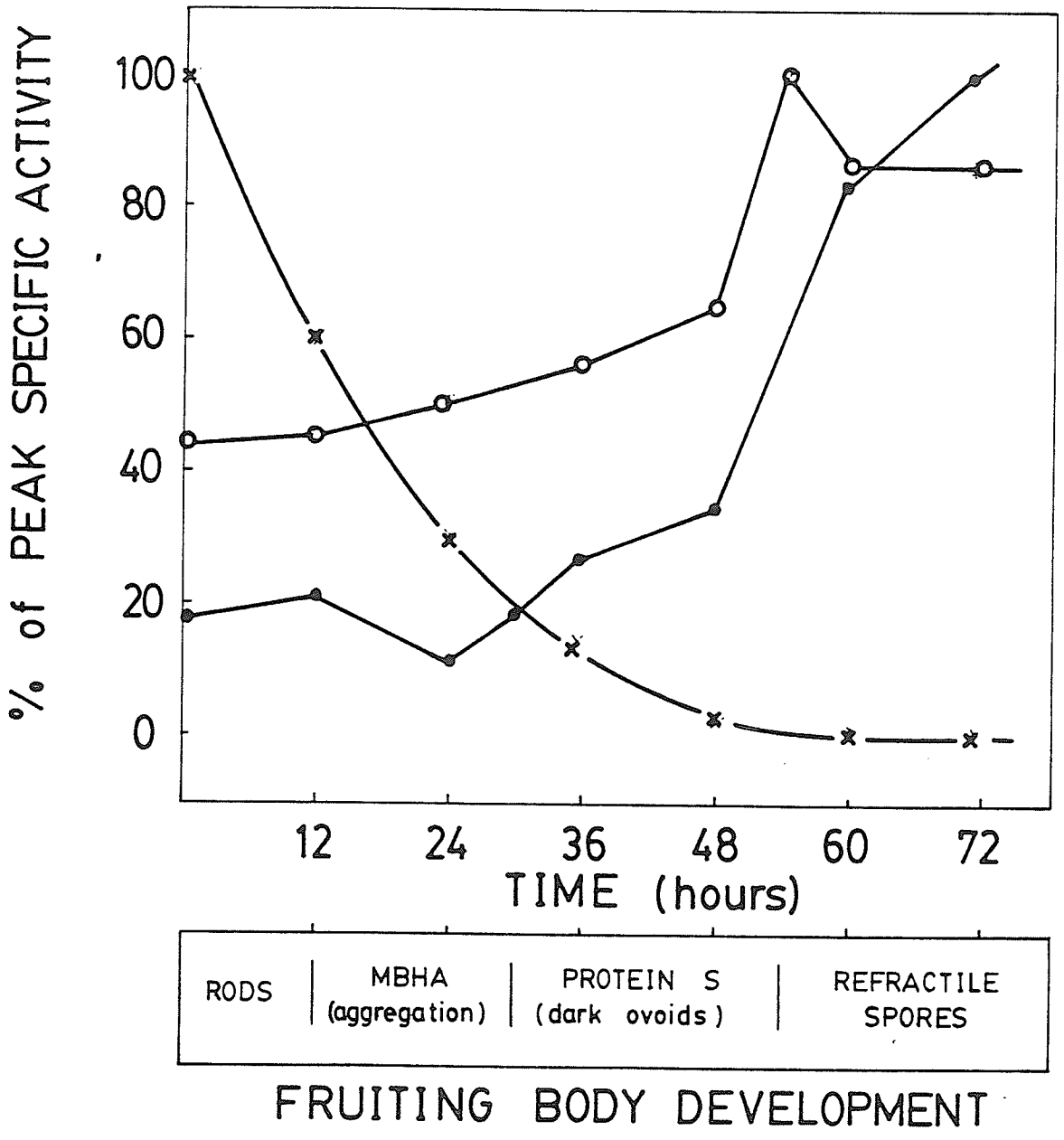
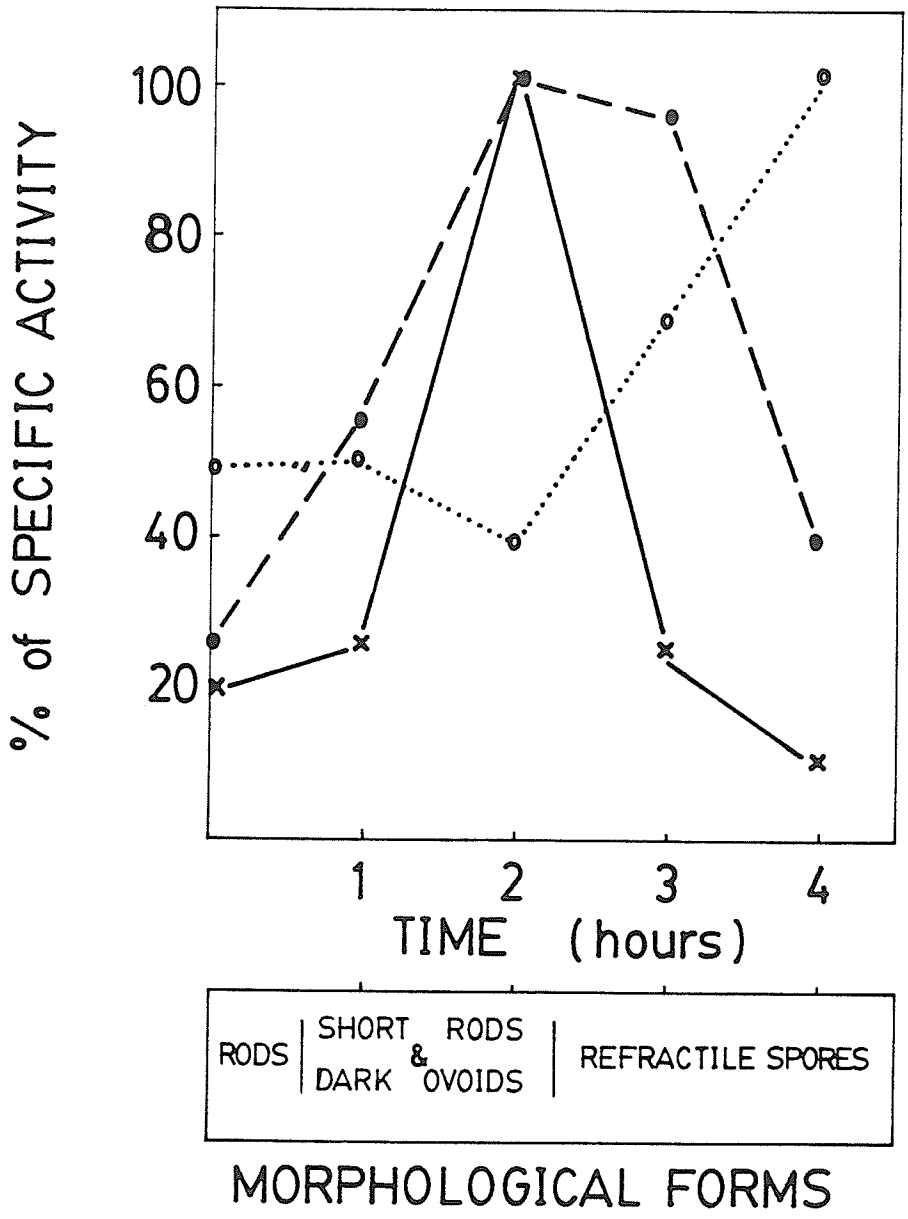

% of PEAK SPECIFIC ACTIVITY
100
80
60
40
20
0
12
24
36
48
60
72
TIME (hours)
RODS
MBHA
(aggregation)
PROTEIN S
(dark ovoids)
REFRACTILE
SPORES
FRUITING BODY DEVELOPMENT

Figure 14. Kinetics of the 3 enzymes during glycerol induction.

Specific activities are expressed as percentages of the peak specific activity of the corresponding enzyme. The peak specific activities were 0.68 μmoles glyoxylate phenylhydrazone formed min/mg protein for isocitrate lyase, (⊸), 0.205 μmoles p-nitrophenol formed/min/mg protein for alkaline phosphatase, (⊸), and 0.1 μmoles UDP-N-acetylglucosamine formed/min/mg protein for UDPG pyrophosphorylase, (⊸) (Filer et al., 1977b).


% of SPECIFIC ACTIVITY
100
80
60
40
20
1
2
3
4
TIME (hours)
RODS
SHORT RODS & DARK OVOIDS
REFRACTILE SPORES
MORPHOLOGICAL FORMS

DISCUSSION

DISCUSSION

Studies on changing levels of enzymes in M. xanthus have been mainly done with cells undergoing induced morphogenesis in liquid culture. However, this system may not necessarily reflect the natural, regulatory and developmental processes observed in Myxococcus since it bypasses the distinctive features of aggregation and fruiting. This study was undertaken to determine the behaviour of 3 enzymes during fruiting body formation with the hope that the activities of the enzymes will prove to be regulated in concert with the total developmental program.

The purified myxospore coat consists of 50% galactosamine (N-acetylated), 25% glucose, 14% protein and 7-8% glycine (Kottel et al., 1975). The increased activity of isocitrate lyase and UDP-glucose pyrophosphorylase at the time of myxosporulation in mature fruiting bodies and in glycerol induced cultures was therefore not surprising (Figs. 13, 14). The high UDP-glucose pyrophosphorylase activity found between 48 and 72 hours of development (Fig. 12) was correlated with the completion of aggregation and the onset of myxospore formation. In glycerol induced cultures increased activity of this enzyme was related to increases in the rate of synthesis of UDP-N-acetylgalactosamine and its precursors (Filer et al., 1977b). UDP-N-acetylgalactosamine is a major precursor of the myxospore coat polymer and the increased enzyme activity probably indicates that cells synthesized their coat polysaccharides during this period.

Isocitrate lyase activity was highest during the same period (Fig. 13)

indicating that the enzyme was required for spore formation, but not aggregation. The increased activity of this enzyme indicates the glyoxylate cycle was operating during this period. The glyoxylate cycle may be required to provide glyoxylate for the synthesis of glycine, a major component of the myxospore coat. As well, the glyoxylate cycle converts material degraded via acetyl coA e.g. proteins and lipids that may arise from lysed cells, to substrates that can eventually enter the gluconeogenesis pathway. The latter is important during this stage for synthesis of sugars required in myxospore polysaccharides. Since the organism is not known to possess glycogen or starch reserves and cannot use sugars as carbon sources, the conversion of amino acids to sugars via the glyoxylate cycle and gluconeogenesis is highly important. As noted in Results, refractile spores first appeared after 54 hours and increased in number until 72 hours of development. The extended period of high enzyme activity coincided with the time when refractile spores were formed. Essentially, the 2 enzymes behaved in the same manner, showing highest activities during acquisition of refractility (Fig. 13).

The results obtained during glycerol induced morphogenesis have been reported previously (History) and correlated to myxospore formation. The peak in isocitrate lyase and UDP-glucose pyrophosphorylase activities at 2 hours of induction coincided with the time when myxosporulation was completed. The activities of isocitrate lyase and UDP-glucose pyrophosphorylase began to increase slowly when rods changed to dark ovoid cells and more dramatically as spores became refractile (Fig. 14).

The activity of the 2 enzymes remained at high levels for a longer period of time during fruiting body formation, in contrast to the rapid decrease in activity observed after glycerol induction. This may be due to the asynchronous and slower conversion to spores within the fruiting body as compared to the rapid, synchronous conversion observed during glycerol induction (Fig. 2). Essentially, the same results were obtained during spore formation in fruiting bodies, indicating that the increase in activities of the two enzymes were associated with conversion of rods to spores rather than with aggregation. Furthermore, the increased activities could serve as markers for these events.

Germination of myxospores could take place in distilled water provided orthophosphate was present or if the spore density was sufficiently high. It was suggested that orthophosphate excreted by spores triggers germination and, in distilled water, a high cell density was required to increase orthophosphate to levels sufficiently high for germination (Dworkin, 1973). Filer et al., (1977b) noted that the activity of alkaline phosphatase extracted from glycerol-induced myxospores was high and that this decreased in extracts of germinating cells. It was suggested that the function of the enzyme was to produce orthophosphate from various organic phosphoesters. The orthophosphate in turn would facilitate germination. The increased activity of alkaline phosphatase in glycerol-induced cells (Fig. 9) supports this hypothesis and suggests that spores prepare themselves for germination by raising their alkaline phosphatase content.

However, in direct contrast, alkaline phosphatase activity could not be detected in myxospores from fruiting bodies, and the activities decreased sharply throughout development (Fig. 10). The reason for this difference is not known. It is possible that during fruiting body formation, but not glycerol induction, an inhibitor, that cannot be removed by the extraction procedure, inhibits enzyme activity. A parallel for this has been reported for alkaline phosphatase activity in developing D. discoideum (Gezelius and Wright, 1965).

Another possibility is that fruiting body myxospores use orthophosphate, derived from polyphosphates, to facilitate germination. It is known that M. xanthus is able to produce these polymers (Voelz et al., 1966). In this case, polyphosphatase, rather than alkaline phosphatase might accumulate during fruiting body formation. Whatever the reason for the discrepancy, the results show there are metabolic differences in the 2 processes. The rapid decrease in alkaline phosphatase accurately marks the onset of the developmental program.

As more is learned about the biochemical changes during myxobacterial development, it will be interesting to compare them with those changes which occur during development of the cellular slime mold, D. discoideum. There are already a number of similarities between the two organisms. For example, they both undertake a similar life cycle involving aggregation and fruiting body formation and they both elaborate a similar system of cell cohesiveness involving lectins (History). Perhaps when more studies are done on the myxobacteria, these 2 systems will prove to be an example of convergent evolution in nature.

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