THE UNIVERSITY OF MANITOBA

Incorporation of ^{32}Pi into Nucleotides, Polyphosphates and Other Acid-Soluble Compounds by *Myxococcus xanthus* During Myxospore Formation.

by

Rand A. Shipman

A Thesis

Submitted to the Faculty of Graduate Studies in partial fulfilment of the requirements for the degree of M.Sc.

Department of Microbiology

Winnipeg, Manitoba

June, 1981.

INCORPORATION OF ^{32}Pi INTO NUCLEOTIDES, POLYPHOSPHATES AND OTHER ACID-SOLUBLE COMPOUNDS BY *MYXOCOCCUS XANTHUS* DURING MYXOSPORE FORMATION

BY

RAND A. SHIPMAN

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

ABSTRACT

Incorporation of ^{32}Pi into Nucleotides, Polyphosphates and Other Acid-Soluble Compounds by *Myxococcus xanthus* During Myxospore Formation.

by Rand A. Shipman

When glycerol was used to induce myxospore formation in *Myxococcus xanthus* in the presence of ^{32}Pi, the label was incorporated into a variety of acid-soluble compounds. Incorporation into ribonucleotides was approximately five-fold greater than in vegetative cells or noninducible mutants grown in glycerol. The label was also incorporated into some unknown compounds and material tentatively indentified as guanosine tetraphosphate. Marked accumulation into polyphosphates, which were present mainly in culture supernatants, occurred relatively late during myxospore formation. The kinetics of accumulation of some of these compounds and their distribution into acid-soluble cell extracts and culture supernatants are described and compared with those in vegetative cells and noninducible mutants.

To my wife, Pat and my son, Paul and my parents.

ACKNOWLEDGEMENT

The author wishes to express his gratitude to his supervisor, Dr. P. Y. Maeba for his support and guidance during the course of this undertaking.

ABBREVIATIONS

ADP	-	Adenosine diphosphate
AMP	-	Adenosine monophosphate
ATP	-	Adenosine triphosphate
cAMP	-	cyclic adenosine monophosphate
CTP	-	cytosine triphosphate
dATP	-	deoxyadenosine triphosphate
dCTP	-	deoxycytosine triphosphate
dGTP	-	deoxyguanosine triphosphate
DNA	-	deoxyribonucleic acid
dNTP	-	deoxynucleoside triphosphate
dTTP	-	deoxythymidine triphosphate
G4P	-	guanosine tetraphosphate
HPN	-	highly phosphorylated nucleo
PEI	-	poly-(ethylene)-imine cellulose
Pi	-	inorganic phosphate
Poly P	-	polyphosphate
ppApp	-	adenosine tetraphosphate
ppAppp	-	adenosine pentaphosphate
ppGpp	-	guanosine tetraphosphate
ppGppp	-	guanosine pentaphosphate
RNA	-	ribonucleic acid
rRNA	-	ribosomal RNA
tRNA	-	transfer RNA

TABLE OF CONTENTS

LIST OF FIGURES

HISTORICAL

The Myxobacterales

The gliding bacteria, Myxobacterales, are a unique and interesting group of bacteria. Their ability to form fruiting bodies and their unusual gliding motility, distinguishes them from other bacteria. The Myxobacterales are characterized by three principle features (Dworkin, 1966): (a) Cells are non-flagellated, but vegetative rods move by gliding over the solid surface of a substrate. (b) The organisms undergo a life cycle involving cellular aggregation of the vegetative rods followed by the formation of fruiting bodies. Within the fruiting body, vegetative rods enter a resting state either by conversion to microcysts or by inclusion within a macrocyst. (c) Myxobacteria are able to hydrolyse insoluble macromolecules. In nature, the myxobacteria are found in soil, on decaying vegetation, the bark of living trees, and animal feces.

Generally, the Myxobacterales are unicellular, slender rods ranging from 0.2 - 1.2 um in diameter and 2.0 - 20.0 um in length which are typically enveloped in a copious slime layer. Gram-negative and quite slender, the cells are capable of a slow gliding motility on solid surfaces or on air-water interfaces (Bergey's Manual 8th Ed.). The vegetative cells are not significantly different, either chemically or in ultra structure, from other Gram-negative bacteria (Voelz and Dworkin, 1962, White et al., 1968).

Strictly aerobic, these chemoorganotrophs employ only respiratory metabolism. They degrade such macromolecules as proteins, nucleic acids, fatty acid esters and various polysaccharides. Characteristically, carotenoid pigments and melanin pigments are often produced. Most species of the myxobacteria will not grow or grow poorly on the usual bacteriological media. The bacteriolytic myxobacters, which includes Myxococcaceae, Archangiaceae, Cystobacteraceae, and most of the Polyangiaceae, may be cultured on agar media containing living or killed bacteria or yeast as nutrient sources. Some, however, will also grow well on medium containing protein hydrolysates and salts. Information regarding the minimal nutritional requirements of the bacteriolytic myxobacters has been determined. According to Dworkin (1962), Hemphill and Zahler (1968) and McCurdy and Khouw (1969), the organisms demonstrate a requirement for complex amino-acid mixtures and relatively high concentrations of Mg^{2+} or Ca^{2+} for growth. Most species show no definite vitamin requirements. The vegetative colony of myxobacters is often referred to as a sworm, or pseudoplasmodium. Slime production and the gliding movement of cells results in the characteristic colony morphology, i.e. flat and thin with many concentric folds and/or rediating lines.

Depending upon environmental conditions, such as poor nutrient levels, the cells may form aggregation complexes. These complexes, in turn, give rise to fruiting body structures which are constructed of slime and cells. The cells

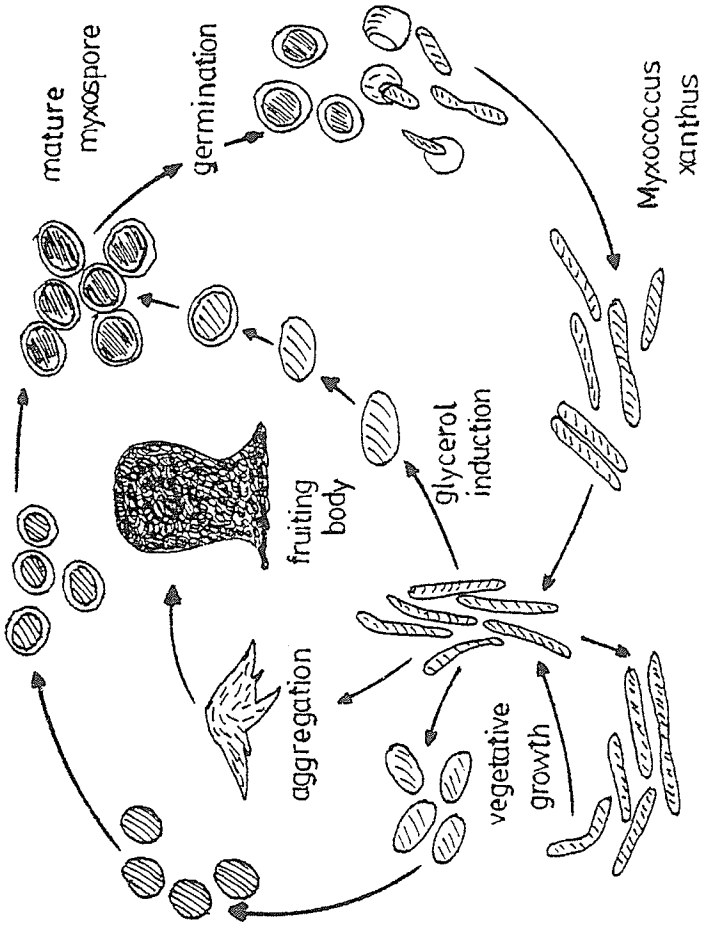


FIG. 1 The life cycle of Myxococcus xanthus.

within these structures become resting cells, called myxospores or microcysts. The hardened slime capsule surrounding the microcyst confers far greater resistance to heat, dessication, physical disruption and ultraviolet radiation than is demonstrated by the vegetative cells. It is on the basis of fruiting body and myxospore morphology, that the taxonomy of the individual families is based.

The fruiting bodies of the myxobacteria vary greatly in morphology. These unusual structures can be simple mounds formed by Myxococcus, or elaborate tree-like structures formed by Polyangiaceae. Microcysts, found within the fruiting bodies, may occur in undifferentiated masses, as in Myxococcaceae, or in cysts, as in Polyangiaceae. Some myxobacteria enclose the vegetative rods within a macrocyst, which serves as the unit structure of the resting stage. This represents an additional and more complex dimension of the development cycle of the myxobacteria. Little is know regarding the physiology and biochemistry of myxospore formation or the internal organization of the fruiting body. It is generally accepted that high nutrient concentrations, while allowing for vegetative growth, inhibit fruiting body formation. Dworkin (1963) has demonstrated that this inhibition is due to the presence of specific inhibitors of fruiting body formation such as phenylalanine and tryptophan.

Sporulation involves the direct conversion of a single vegetative cell to single myxospore by gradual enlargement, and shortening of the rod in the direction of its longi-

tudinal axis, resulting in a spheroid structure. Upon maturation, this structure becomes optically refractile. Changes in fine structure during microcyst formation in Myxococcus xanthus have been followed by Voelz and Dworkin (1962). Electron microscopy revealed that there was no thickening or organizational change of the cell wall, but there was, instead, an accumulation of a very thick spore coat.

Upon germination of the microcysts, there is a slight swelling followed by a loss of refractility. The coat of the microcyst is then penetrated from within by a short thick rod which elongates, leaving behind an empty shell. This shell consists of both capsular and wall material of the microcyst. The emergence of the vegetative cell is preceeded by a shrinking away of the protoplast from the wall-capsule complex, followed by a localized rupture of the wall capsule.

Myxococcus xanthus:

M. xanthus is the best characterized species of myxobacteria, due to the relative ease with which it can be cultured. Its life cycle resembles that of the eukaryotic slime mold, Dictyostelium discoideum. When cells on solid medium are deprived of specific nutrients, they shift from growth to development (differentiation). They migrate by means of their peculiar gliding motility to aggregation centres to give rise to fruiting bodies. Cells inside the fruiting body develop into myxospores, which are optically

refractile, resistant, resting cells. The myxospores remain dormant until favorable nutritional conditions are encountered, in which case germination is stimulated. Figure 1 (Wireman and Dworkin, 1975) details the life cycle of M. xanthus.

Burchard and Dworkin (1966a) have reported two colony types which arose when M. xanthus FB (Fruiting Body) was plated out on nutrient plates. These have been designated as "yellow" and "tan" colony types, based on the presence of a yellow pigment in "yellow" colonies and the lack of it in "tan" colonies. Burchard (1974) later reported morphological variants, which arose spontaneously when M. xanthus FB was plated out. These have been denoted as: YS (Yellow Swarmer), TS (Tan Swarmer), YNS (Yellow Non-Swarmer), and TNS (Tan Non-Swarmer). Although the four colony types appear spontaneously, conversion from one type to another can be induced or accelerated by either physical or chemical methods. (Sudo and Dworkin, 1969; Burchard, 1975).

Chemical Induction of Sporulation in M. xanthus:

It is possible to induce myxospore formation directly in liquid culture, by-passing the normal developmental formation of fruiting bodies (Dworkin and Gibson, 1964 ; Dworkin and Sadler, 1966a). This was achieved by adding to log phase cultures compounds having primary or secondary alcohol groups such as glycerol, ethylene glycol, butanol and phenethyl alcohol, or the reagent dimethyl sulfoxide. Under these

conditions, vegetative cells differentiated into myxospores rapidly, quantitatively and relatively synchronously. Vegetative rods progressively shortened and became more spherical and within 100 to 120 minutes, they had become round, optically refractile, resistant resting cells similar to myxospores found in fruiting bodies (Figure 1.).

During the induction process (Dworkin and Sadler, 1966a), the optical density of the culture rose immediately after glycerol addition, but within a minute or so, it dropped slightly below the original value. This was followed by an interval of 10 to 15 minutes, when there was no change in turbidity followed by a linear decrease in turbidity that levelled off at approximately 60 minutes. After a stable period of about 15 minutes, turbidity showed a slow linear increase. No morphological changes were observed until 30 to 40 minutes had elapsed when rods began to shorten. At approximately 60 minutes the rods were clearly ovoid, and these became refractile over the next 50 to 60 minutes. The conversion was quite synchronous and approximately 100% of the cells formed microcysts.

For glycerol-induced microcyst formation, either Ca^{2+} (absolutely required in defined media) or Mg^{2+} were required. For optimum conversion, 0.5M glycerol was added to mid log cultures under good aeration at a temperature of 30°- 35° C. If the cells were centrifuged, washed and resuspended in fresh medium lacking glycerol, they returned to the vegetative state.

Macromolecular and Morphological Changes Accompanying Sporulation:

During glycerol induction of myxospores, there were substantial changes in macromolecular syntheses. These changes were directed towards: (1) The structural conversion of the cells. (2) The conversion to state of metabolic quiescence. (3) Preparation for subsequent germination (Wireman and Dworkin, 1975).

The overall pattern of macromolecular synthesis during myxospore formation has been examined by Sadler and Dworkin (1966b). The RNA, DNA and protein contents of the microcyst increased 20% to 35% over that of vegetative cells. Since most and probably all stages of the conversion process were sensitive to both chloramphenicol and actinomycin D (Dworkin and Sadler, 1966b), it has been suggested that synthesis of RNA and protein were absolutely required for microcyst development. This had been substantiated by the fact that if the two processes were blocked, no further development took place.

Rosenberg et. al (1967) have suggested that, with the initiation of myxospore formation, DNA synthesis continued until all exisiting rounds of replication had been completed with no new rounds being initiated. In another study (Bacon and Rosenberg, 1967), it was observed that although net RNA synthesis ceased immediately after induction, an increased turnover of RNA synthesized both before and after induction took place and ribosomes and ribosomal RNA were synthesized

during microcyst formation. Also, net protein synthesis continued during the morphological conversion. Two hours after induction, net protein synthesis ceased, accompanied by a high rate of continuous protein turnover (Orlowski and White, 1974).

The appearance of the spore coat is one of the striking changes in cellular structure during myxospore morphogenesis. White (1975) has determined that this structure is largely carbohydrate and approximately 50% of the coat dry weight was N-acetyl glucosamine.

Recently, attention has shifted to studying colonial morphongenesis on solid medium rather than cellular morphogenesis, i.e to the processes of swarming, aggregation and the formation of fruiting bodies. The complex nature and precise organization of these processes suggest that systematic cell interactions play a dominant role in myxobacterial development (Wireman and Dworkin, 1975).

Time-lapse photomicroscopy studies have led to the conclusion that the myxobacteria are group orientated during all stages of their life cycle and grow characteristically as swarmers. An individual cell on the edge of the swarm remained in close contact with the group. Although outward explorations did occur, cells quickly returned to the swarm. During fruiting body formation, cooperation between cells was even more noticeable. There were distinct streams of cells moving into aggregation centres, often accompanied by rythmic oscillations around the aggregation centres (Shimkets and

Seale, 1975 ; Hodgkin and Kaiser, 1977).

These behavioral patterns are much akin to fruiting body construction in the cellular slime mold, Dictyostelium discoideum. However, the aggregation to form "slugs" and the migration of the "slugs" are morphologically distinct developmental stages which are not observed in myxobacterial development (Wireman and Dworkin, 1975).

Enzymatic Changes Accompanying Sporulation:

During glycerol induction of myxospores, a number of enzymatic activities have been shown to change.

Watson and Dworkin (1968) examined some of the enzyme activities associated with crude extracts of both vegetative cells and glycerol-induced microcysts of M. xanthus. In assaying the enzymes required for glycolysis, gluconeogenesis and the tricarboxylic acid cycle, only isocitrate dehydrogenase was found in higher concentrations in induced microcysts than in vegetative cells.

Orlowski et.al (1972) examined the activities of isocitrate lyase and malate synthase in extract of M. xanthus from different stages during myxospore formation. Both enzyme activities reached peak values during the conversion of vegetative cells to microcysts then declined; the isocitrate lyase activity declining much more rapidly than that for malate synthase. A later study by Orlowski and White (1974) examined the inactivation of isocitrate lyase, which occurred in the later stages of myxospore development. Protein

synthesis was required at a specific interval in order for inactivation to occur at a later time. Metabolic energy was also required at all times during myxospore development if the inactivation was to occur.

Recently, Filer et.al., (1977a, 1977b) have examined the enzymes associated with the formation of uridine-5'-diphosphate-N-acetylglucosamine, a critical intermediate for spore coat synthesis. Examination of cell extracts obtained at different stages of myxospore development, revealed there was a 4.5 to 7.5 fold increase in specific activity of these enzymes after 2 h of induction. Two to 4 h after the onset of microcyst germination, the levels decreased to those normally associated with vegetative cells.

Several studies have shown that the deletion of specific required amino acids from defined agar medium stimulated fruiting body formation (Dworkin, 1963; Hemphill and Zahler, 1968). Witkins and Rosenberg (1970) showed that myxospore formation was induced on defined medium in which methionine was absent or had been replaced with threonine and lysine. Subsequently it was shown that the aspartate family of amino acids exert an influence on fruiting body formation which could be correlated with their effect on aspartokinase. Aspartokinase catalyzes the first reaction in the pathway giving rise to L-lysine, L-isoleucine, L-methionine, L-threonine and α-ε-diaminopimelic acid. This enzyme in M. xanthus was repressed and feedback inhibited by L-lysine and L-threonine and "feedback-stimulated" by L-isoleucine and L-methionine.

Methionine and isoleucine stimulated aspartokinase activity *in vitro* and inhibited fruiting. Threonine and lysine inhibited aspartokinase and stimulated fruiting (Wireman and Dworkin, 1975; Filer et.al., 1973). Rosenberg has put forth the hypothesis that the decrease in aspartokinase activity results in starvation for α-ε-diaminopimelic acid, and the resulting blockage of vegetative all wall synthesis leads to induction of the developmental cycle. This was substantiated by the fact that DAP synthesis was required for cell growth but there was no net increase in the level of DAP during myxospore formation (White et.al., 1968).

Campos and Zusman (1975) constructed a defined medium that allowed *M. xanthus* to grow vegetatively just above the threshold of starvation and fruiting. Using this "limited" medium, it was possible to determine the effect of various chemicals on fruiting. It was found that cAMP elicited extensive fruiting. Control plates lacking cAMP did not display any fruiting bodies. In the absence of cAMP, fruiting body formation was stimulated by starvation and inhibited by casitone. Although cAMP alone could stimulate fruiting at nutritional levels near the threshold of starvation, other adenine nucleotides also proved stimulatory: ATP, 2'-AMP, 3'-AMP, 5'-AMP and noteably ADP. The most effective stimulus for fruiting, ADP, was also a potent inhibitor of aspartokinase activity *in vitro* (Filer et.al., 1973). Threonine, which alone, stimulated fruiting, synergistically enhanced fruiting when added along with ADP or cAMP. Methionine or

isoleucine, which alone inhibit fruiting, abolished the stimulation by ADP or cAMP.

The reports from Campos and Zusman (1975), Filer et.al., (1973), and Rosenberg et.al., (1973) suggest that initiation of fruiting body formation in M. xanthus was subject to multiple controls (i.e. nutritional and biochemical). The stimulation by the adenine nucleotides may therefore reflect a mechanism controlling development that is related to nutrient deprivation, possibly through release from catabolite repression of developmental cistrons (Schwartz et.al., 1970).

Fruiting body formation showed a strong dependence on the initial cell density. Cells plated out at high densities form fruiting bodies rapidly and synchronously with no net vegetative growth. At lower cell densities, no fruiting bodies appeared although enough cells were present. McVittie and Zahler (1962) showed that a diffusable chemical was involved in fruiting, but it is unknown whether a minimum number of cells was required to initiate chemical signaling.

Germination, another developmental process, is also dependent on cell density. Glycerol induced myxospores of M. xanthus germinated in distilled water, if they were present at a high cell density. However, this cell density-dependent germination could be overcome by the addition of orthophosphate to the cell culture. Ramsey and Dworkin (1968) have shown that myxospores suspended in distilled water excrete orthophosphate, at a concentration sufficient to serve as a germination trigger.

Highly Phosphorylated Nucleotides and Nucleotides Involved in the Regulation of Sporulation (Development) in Other Organisms:

It is generally accepted that most cells can coordinately regulate a large variety of physiological activities in response to nutrient levels. The bacterial response to limitation of amino acids is perhaps the best studied of these mechanisms. Sands and Roberts (1952) and Pardee and Prestidge (1956) first noted the drastic reduction of stable RNA synthesis when cells were starved for amino acids. This was later termed the "stringent response" by Stent and Brenner (1961). Mutants that continued to synthesize RNA in the absence of protein synthesis, i.e when starved for amino acids, were termed "relaxed" (Borek et.al., 1956).

Cashel and Gallant (1969) found that amino acid starvation in stringent strains of Escherichia coli caused the accumulation of two guanine nucleotides, guanosine 5'-diphosphate 3'-diphosphate (ppGpp, MS I) and guanosine 5'-triphosphate 3'-diphosphate (ppGpp, MS II). These compounds were absent from "relaxed" strains of E. coli. This led to the formation of the hypothesis that high intracellular concentrations of these compounds caused a cessation of RNA accumulation and to other physiological changes characteristic of the stringent response. Since being characterized (Cashel and Kalbacher, 1970), MS I has been shown to regulate RNA synthesis in a number of microorganisms. In E. coli stringent strains accumulation of ppGpp led to reduced synthesis of rRNA (Reiness et.al., 1975; Nierlich, 1968; Lazzarini and

Dahlberg, 1971), tRNA (Primakoff and Berg, 1970; Ikemura and Dahlberg, 1973) and some species of mRNA (Nierlich, 1968; Lazzarini and Dahlberg, 1971).

Reiness et.al., (1975) examined the effect of ppGpp on the expression of genes, other than the rRNA genes. They observed no selective reduction of tRNA synthesis, whereas the tryptophan and lactose operons were stimulated by this nucleotide. It was hypothesized that ppGpp interacts with RNA polymerase to alter the enzyme's affinity for promotors in an operon specific manner.

MS I has also been observed in some eucaryotic cells. Klein (1975) reported its presence during early stages of starvation-induced differentiation in Dictyostelium discoideum. Rhaese (1975) observed its presence in some lines of cultured mammalian cells and Irr et.al., (1974) demonstrated that ribosomes from rat embryos, but not adults, could synthesize ppGpp from GTP in the presence of ATP, in vitro. Haseltine et.al., (1972) suggested that its formation resulted from an "idling" step in protein synthesis when cells are starved for amino acids.

Besides the two MS compounds, other highly phosphorylated nucleotide have also been found. Rhaese and his co-workers, studying mammalian cells and Bacillus subtilis, have observed four and possibly six highly phosphorylated nucleotides (HPN) (Rhaese, 1975; Rhaese and Groscurth, 1974). These compounds have been designated HPN I, HPN II, HPN III and HPN IV. Rhaese et.al., (1972) reported that at the start of sporulation in

B. subtilis, HPN I, HPN II and HPN III accumulated within the cells, while HPN IV was excreted into the medium. MS compounds were produced in response to amino acid starvation whereas synthesis of the HPN nucleotides was in response to depletion of carbon sources (Rhaese et.al., 1975; Galland and Morgason; 1972). Once protein turnover began, due to protease activity, the levels of MS I and MS II decreased and HPN III and HPN IV levels began to increase. Rhaese and Groscurth (1974) have shown that ribosomes of vegetative B. subtilis synthesized MS I and MS II but not HPN I or HPN II, and that ribosomes of sporulating cells synthesized little or none of the MS compounds but synthesized HPN I and HPN II. The latter two compounds were identified as adenosine 5'-diphosphate 3'-diphosphate (ppApp) and adenosine 5'-triphosphate 3'-diphosphate (pppApp), respectively. Swanton and Edlin (1972), using a mutant of B. subtilis, demonstrated that MS I and MS II were not necessary for differentiation. However, HPN III and HPN IV arose only during sporulation.

Gallant, Shell and Bittner (1976) reported the existence of another novel nucleotide that has been implicated in the response of E. coli to an energy source downshift. This compound, called the "phantom spot", decreased markedly within a minute of downshift, which correlated well with the adjustment of RNA accumulation.

The levels of nucleoside triphosphates also change with the environmental conditions. These conditions also influence the rate of growth as well as the rate of synthesis and the

levels of stable RNA species. Constantini et.al., (1977) examined the levels of the four ribonucleoside triphosphates (ATP, CTP, UTP, GTP) in Neurospora crassa under conditions of exponential growth as well as shift-up and shift-down transitions of growth. The nucleotide pools, in some cases, appeared proportional to the rate of synthesis of rRNA, although this seemed not to be strictly dependent on the level of the nucleotides. Similar findings have been reported for E. coli (Sturani et.al., 1976; Sturani et.al., 1973) and Bacillus megaterium (Singh et.al., 1977).

Polyphosphates

The isolation and characterization of various polyphosphates and unique phosphorylated nucleosides, produced during altered growth conditions, have led to suggestions that they may be possible regulators of growth in some procaryotes and eucaryotes. Wiame (1947; 1948) and Schmidt et.al., (1946) first isolated characterized polyphosphates (poly P) from yeast. Since then, poly P have been isolated from a vast number of microorganisms, higher plants and animals. Between different organisms, Harold (1966) noted a considerable heterogeneity in the size and quantity of endogenous poly P. Contrary to this, poly P with chain lengths of 3, 4, 10 and 300 have been isolated from Saccharomyces cerevisiae. Although a great deal of data has been collected regarding poly P metabolism, little is known about its biological functions. Physiological studies, however, related to poly p metabolism have shed some light as

to its significance.

A common observation is the large fluctuation in intracellular pool sizes of poly P which itself is dependent on the growth conditions. Poly P levels are low in organisms undergoing rapid growth while elevated levels prevail during conditions of nutritional imbalance, resulting in growth inhibition (Harold, 1966). In bacteria, poly P accumulation was elicted by starvation for nitrogen (Sall et.al., 1956), sulfer (Harold, 1966) and zinc (Winder and O'Hara, 1962) starvation. Related to nitrogen starvation was the inverse relationship between nucleic acid biosynthesis and poly P levels in Aerobacter aerogenes (Harold, 1966). Owing to the fact that upon resumption of growth and concomitant degradation of poly P, there is incorporation of released Pi into nucleic acids, it has been suggested (Harold, 1966) that poly P may serve as a phosphate storage during periods of non-growth.

In yeast and filamentous fungi, similar physiological properties have been noted. In Asp. niger, large amounts of poly P were found in the spores. During spore germination, poly P was degraded by polyphosphatases to Pi, and incorporated into phospholipid and nucleic acid (Nishi, 1960; Nishi, 1961). Goodman et.al., (1969) reported that, in Physarum polycephalum, the level of poly P was low during periods of active RNA and DNA synthesis and high during differentiation (spore formation). Hildebrandt and Sauer (1977) proposed that insoluble poly P may be involved in the differentiation

process of this organism by specifically inhibiting rRNA synthesis. Ludwig et.al. (1977) observed in Saccharomyces cerevisiae that increased levels of low molecular weight poly P preceeded any increase in the rate of RNA synthesis, which led to the hypothesis that these compounds might serve to trigger an increase in the rate of macromolecular synthesis.

The existence of large amounts of poly P is so many diverse organisms indicates that it may have some important role in cellular regulation. However, research to date has not been unable to confirm this.

The HS Compounds

In 1975, Lé John et.al. reported the presence of unusual phosphorylated nucleotides in acid extracts from a variety of fungi. These compounds were designated as HS 1, HS 2, and HS 3. Characterization of these complex molecules (McNaughton et.al., 1978) revealed that HS 1 and HS 2 were uridine dinucleotides with 12 and 10 phosphates respectively, whereas HS 3 was made up of adenosine, uridine and 8 phosphates.

The studies of Lé John et.al. (1978) implicated these HS molecules as regulators of sporulation and nucleic acid metabolism. For example, it was noted that a rapid intracellular accumulation of all three HS compounds occurred just prior to sporulation. This accelerated increase of HS molecules in Achlya was triggered either by the depletion of phosphate in the growth medium or by transferring the cells from a rich growth medium to one lacking nutrients. These sudden nutritional changes invariably resulted in sporulation. However,

when the starvation medium was amended with glutamine or various purine and pyrimidine bases and nucleosides, HS accumulation and sporulation were simultaneously inhibited. As a result, it was suggested (Lé John et.al., 1978) that the intracellular accumulation of all HS molecules was a prerequisite for sporulation. When the rates of RNA and DNA synthesis were compared to HS synthesis, an inverse relationship was observed (Lé John et.al., 1975; Lé John et.al., 1978). This indicated that HS molecules could be acting as negative effectors of RNA and DNA synthesis *in vivo*. *In vitro* experiments demonstrated that HS 3 and HS 2, at physiological concentrations, were potent inhititors of isolated *Achlya* DNA-dependant RNA polymerases (McNaughton et.al., 1975; Lé John, 1978) and ribonucleotide reductase (Lewis et.al., 1977).

MATERIALS AND METHODS

Organism:

M. xanthus MD-1 obtained from M. Dworkin (University of Minnesota, Minneapolis) were used throughout. Stock cultures were stored at -70°C in casitone growth medium containing 0.5M glycerol.

Growth:

The growth medium consisted of 1% (wt/vol) casitone (Difco) containing 10.0 mM $MgSO_4$. The phosphate concentration in this medium, as determined by the method of Ames (1966), was 0.5 mM. Cells were routinely grown in 40ml of this medium in 250ml Klett flasts which were incubated at 32°C in a gyratory water bath shaker (New Brunswick Scientific Co.) set at 160 rpm. When smaller volumes were required, 5-10ml of the culture were incubated in 50ml Erlenmeyer flasks under the same conditions. Growth of 40ml cultures of *M. xanthus* was monitored as the increase in optical density as measured by a Klett-Summerson colorimeter fitted with a red filter (Fig. 2). The generation time was calculated to be around 3.4 h, which was in agreement with that determined by Wireman and Dworkin (1975).

For myxospore formation, logarithmic phase cells (175-200 Klett units, red filter) were centrifuged at 12,000 xg for 10 min at 4°C (Sorvall RC-5 Superspeed Refrigerated Centrifuge). The cell pellet was resuspended to its original

density in fresh medium and allowed to resume growth at 32°C with shaking. After 60 min, sterile glycerol (6.8 M) was added to a final concentration of 0.5 M and incubation was continued with shaking. For vegetative cell controls, sterile water was added instead of glycerol. Cell and spore counts of cultures, diluted with distilled water, were done microscopically with a Petroff-Hauser bacterial counting chamber (Hausser Scientific Co.).

$^{32}P_i$ - Labelling and Extraction of Labelled Compounds

Orthophosphate, ^{32}P (New England Nuclear), was added to cultures at a concentration of 250-500 uCi/ml 30 min prior to the addition of glycerol or water. Samples (0.5 to 1.0ml) were removed at the appropriate times and transferred to ice-cold centrifuge tubes, then centrifuged at 12,000 xg for 10 min. The supernatant were frozen at -75°C and lyophilized, and the resulting viscous residue was made to 1/5 the sample volume with 0.1 M formic acid (ph 3.0) and analyzed. Identical results were obtained if the residue was dissolved with 1.0 M formic acid. The cell pellet was washed once with ice-cold medium containing 0.5 M glycerol, extracted with 1/5 volume of ice-cold 2.0 M formic acid (ph 3.0) for 30 min, then centrifuged to remove cell debris before analysis (Cashel and Gallant, 1969).

For pulse-labelling, centrifuged cells were allowed to grow for 60 min in fresh medium as previously described. After addition of glycerol to 0.5 M to induce myxospore formation,

Fig. 2 Growth curve of M. xanthus grown in 1% casitone and 0.01 M $MgSO_4$. Temperature 32°C; Aeration 160 rpm; generation time 3 h 35 min.

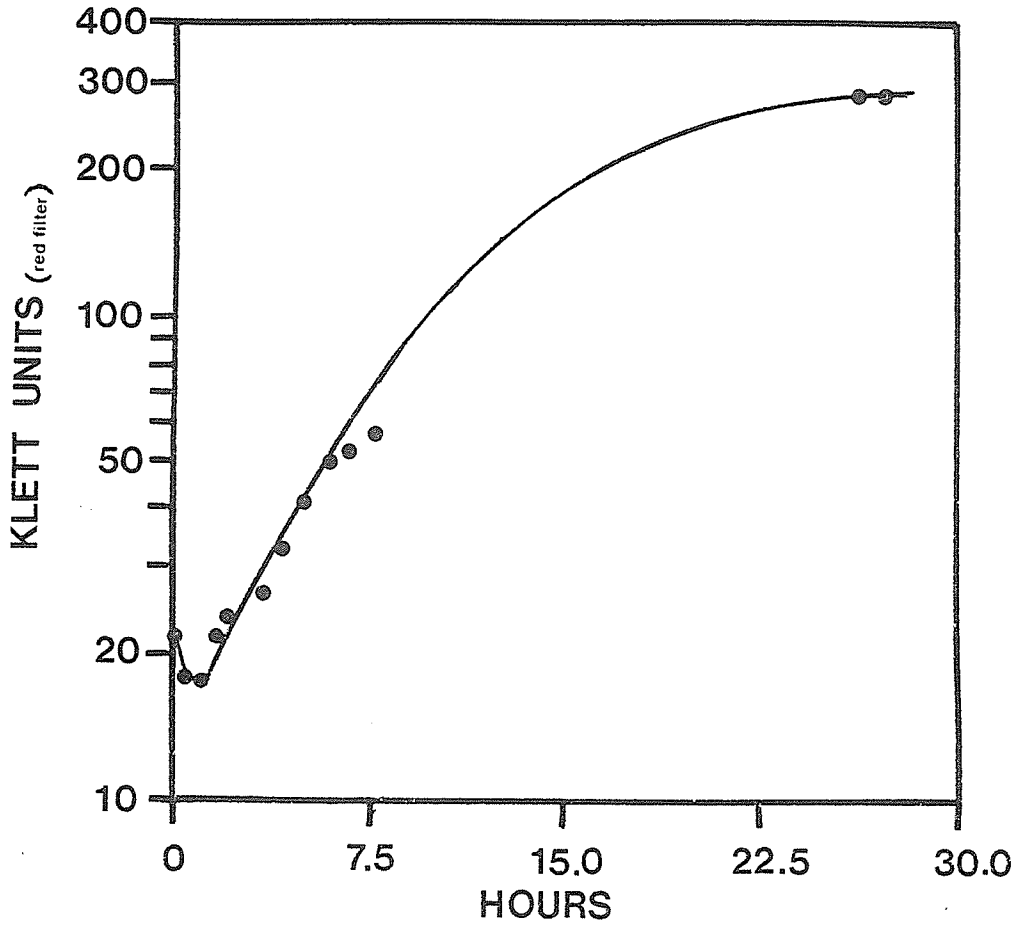

400
300
200
100
50
20
10
KLETT UNITS (red filter)
0
7.5
15.0
22.5
30.0
HOURS

FIG.2

1.0ml amounts were placed in 15.0ml round-bottom centrifuge tubes, and incubation with shaking, was continued at 32°C. As judged by microscopic examination, the time course of myxospore formation was the same as in larger culture. At specified times, 250 uCi of ^{32}P was added to the culture, and, after 30 min of incubation, the culture was cooled in ice, then separated into cells and supernatants, which were extracted as above.

Chromatography:

Commercially available thin layer chromatography plates were washed with distilled water before use. Five to 10 ul aliguots of the extracted samples were spotted on poly-(ethylene)-imine cellulose thin-layer plates (20 by 20 cm, PEI plates; Brinkmann Instruments, Inc.) for chromatography in two dimensions according to the method of Cashel et.al., (1969). The first-dimension buffer was 3.3 M ammonium formate and 0.68 M boric acid adjusted to ph 7.0 with NH_4OH. The second-dimension buffer was 1.5 M KH_2PO_4 (ph 3.65). Prior to running the second dimension, PEI plates were washed in distilled water for 10 min, followed by washing in methanol for 5 min. For one dimensional chromatography, 2 cycles of chromatography with the same buffer was employed. The plates were washed with distilled water followed by methanol between cycles.

After chromatography, the plates were exposed to Kodak RP-1 Xray film (Eastman Co.) for 24-48 h and developed.

Regions on the chromatogram corresponding to the radioactive areas detected on the film were cut out and counted in a Beckman LS230 scintillation counter. The scintillation cocktail consisted of 15.2g 2,5-diphenyloxazole and 0.35g 1.4-bis-[2-(5-phenyloxazolyl)]-benzene per gallon of toluene.

ATP Assay:

ATP was measured by the firefly luciferase assay method of Strehler and Totter (1954). Cells were collected on 25mm diameter membrane filters (0.45um; Millipore Corp.) and extracted in 4.0ml of 0.05 M tris (hydroxymethyl) amino-methane-hydrochloride (ph 7.7) at 100°C for 8 min. Measurements were carried out on a model 2000 ATP Photometer (JRB, Inc.).

Standard nucleotides, used for markers, and enzyme were obtained from Sigma Chemical Co. The "unusual" nucleotides guanosine-5'-diphosphate-3'-diphostate and guanosine-5'-triphosphate-3'-diphosphate were purchased from ICN Pharmaceuticals.

Purification of ^{32}Pi:

In some cases, ^{32}Pi was purified by adsorption to a column of A-25 diethylaminoethyl-Sephadex (Pharmacia) equilibrated in 0.01 M triethyl ammonium bicarbonate (pH 8.0) and eluted with a 200ml linear gradient of 0.01 to 0.5 M triethylammonium bicarbonate. The radioactivity was monitored by Cerenkov counting of aliquots of 1.0ml fractions. The

first peak fractions with radioactivity were pooled, evaporated to dryness under vacuum, and brought to the original volume in distilled water. The preparation was made 1.0 N with concentrated HCl, placed in a boiling water bath for 15 min, then neutralized with 10N NaOH.

RESULTS

As previously mentioned (see Historical), researchers have reported small molecular weight phosphorylated compounds that regulate a number of physiological activities in response to nutritional levels. Because sporulation in Myxococcus xanthus is elicited by the environmental and nutritional conditions, it was thought that regulatory compounds similar to those observed previously may be associated with the process. A search for such compounds was undertaken in M. xanthus that were induced to sporulate with 0.5 M glycerol.

After addition of glycerol to cultures, morphological changes as reported by Dworkin (1964) were observed. As a preliminary test to determine whether unusual acid soluble phosphorylated compounds were produced during glycerol-induced sporulation, ^{32}Pi was added with glycerol to a concentration of 400uCi/ml to 20ml of log phase culture. After 4 h incubation, the cells were harvested by centrifugation and the supernatant fraction, i.e., the spent medium, and cell pellet were extracted with formic acid as described in Methods. The extracts were subjected to 2-dimensional chromatography on PEI plates from which autoradiograms were prepared (Methods). These are shown in Figure 3. The spots labelled G4P, GTP, dGTP, dATP, ATP, GDP, CTP, ADP, dCTP, UTP and dTTP were identified by co-chromatography with unlabelled nucleotides. Their migration was detected under uv light. The faint spot directly to the

Fig. 3. Autoradiograms after two-dimensional chromatography of cell extracts (top left) and supernatant extracts (bottom left) from ^{32}P-labeled cultures (400 uCi/ml). Cultures were induced with 0.5 M glycerol for 4 h before extraction (Methods). The schematic drawing (right) is a composite of the autoradiograms and illustrates the lettering system used to identify the spots that appeared. G4P, Guanosine tetraphosphate. Arrows indicate the direction of chromatography and the buffer system employed.

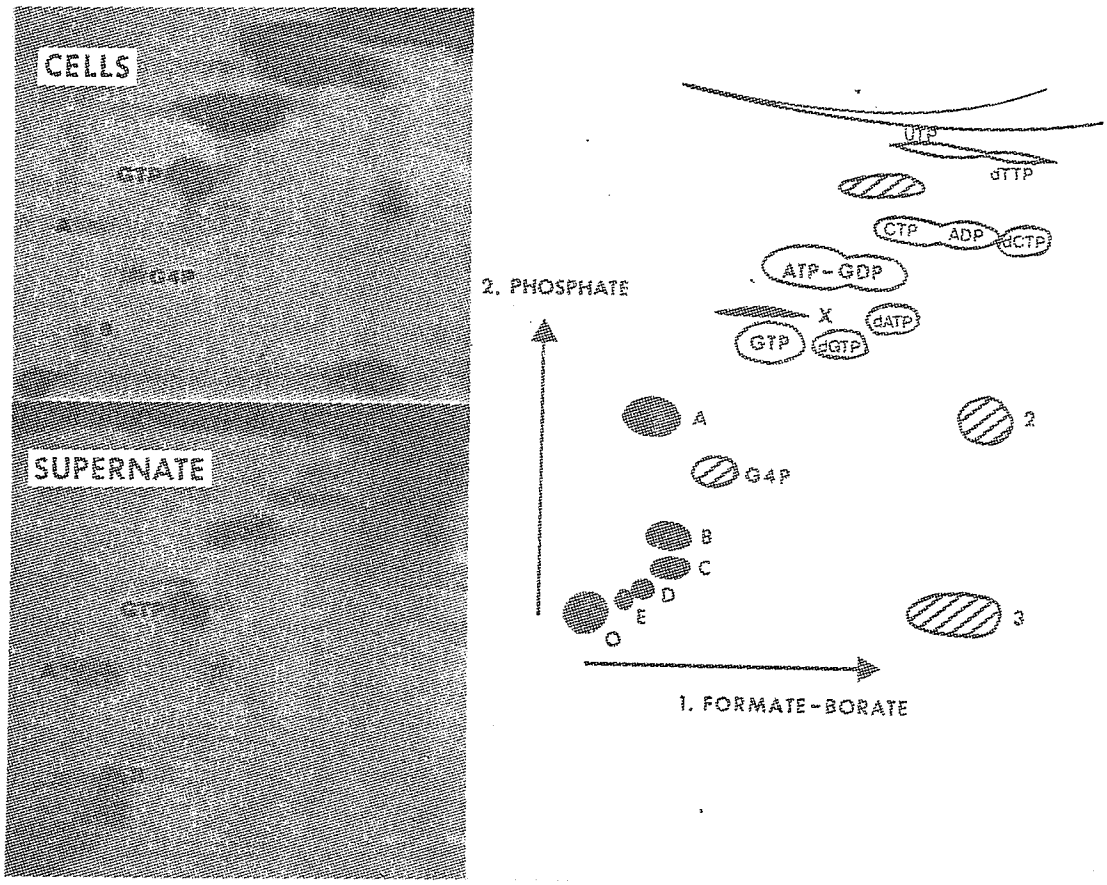

CELLS
GTP
G4P
SUPERNATE
GTP
UTP
dTTP
CTP
ADP
dCTP
ATP-GDP
X
dATP
GTP
dGTP
2. PHOSPHATE
A
G4P
B
C
D
E
O
2
3
1. FORMATE-BORATE

FIG.3

right of GTP was likely dGTP (Cashel, Lazzarini and Kalbacher, 1969). This was later confirmed by co-chromatography. Spots appearing just below GTP were not always observed, and hence were not counted. The diagramatic representation in Fig. 3 (right) indicates the lettering system employed to identify the compounds examined. Since the volumes for cell and supernatant extracts were the same, as well as the sample volumes placed on the chromatographs, the intensity of labelling of the compounds on different chromatograms could be compared. On this basis, the compounds were divided into three groups. One group of compounds labelled more intensely in the cell extracts than in the supernatant fractions and are illustrated as open symbols in the schematic (Fig. 3). These included GTP, ATP, and GDP. The latter two compounds could not be resolved in this system and were counted as one spot, ATP-GDP. The second group of compounds were found in both fractions but labelled much more intensely in the supernatant extracts. These are designated by the solid symbols in the schematic, and include those spots denoted X, A, B, C, D, E and O (Fig. 3). The final group, is indicated by diagonal markings in the schematic and were found solely in cell extracts. These include compounds 2 and 3 and guanosine tetraphosphate (G4P or ppGpp).

Accumulation of ^{32}Pi into Acid-Soluble Compounds:

The accumulation of label into acid-extractable compounds was measured to determine whether its appearance in

the different compounds was related to sporulation. A 20ml culture of log phase cells was centrifuged, resuspended in the same volume of fresh medium and incubated for 1 h at 30°C. ^{32}Pi was added to a concentration of 250uCi/ml and incubation was continued for 30 min, after which sterile gylcerol was added to a final concentration of 0.5 M to initiate myxospore formation. Incubation was continued and 1.0ml samples, removed at various times, were separated into cell pellet and supernatant fractions. These were extracted and subjected to 2-dimensional chromatography as described in Methods. The radioactivity associated with each of the compounds was detected by autoradiography and counted as described in Methods. Samples from a control culture to which water, instead of glycerol, was added were treated in the same way.

Figure 4 summarizes the accumulation of ^{32}Pi into GTP and ATP-GDP. Although radioactivities remained constant in control cells, there was increased accumulation of label into nucleotides of sporulating cultures. Accumulation into cellular nucleotides began 30 min after glycerol addition, whereas accumulation into the supernatant nucleotides commenced 2 h after induction. This suggests that the intracellular nucleotides reach a certain level before excretion into the culture medium occurs. The accumulation of label was generally complete by 3 h, the time at which virtually all (>95%) of the cells appeared morphologically, as myxospores. The accumulation represented a 5-fold increase, approximately, in labelling over that in vegetative cultures.

Fig.4. Accumulation of ^{32}Pi (250 uCi/ml) into ATP-GDP by glycerol-induced cultures (closed symbols). Glycerol or water was added at zero time, 30 min after addition of ^{32}Pi and cell extracts (●, ○) and culture supernatant extracts (■, □) were analyzed (text). The counts shown are relative to the maximum amount incorporated into cell extracts by induced cultures. For ATP-GDP the maximum was 57,000 cpm/10^8 cells, and for GTP it was 30,000 cpm/10^8 cells.

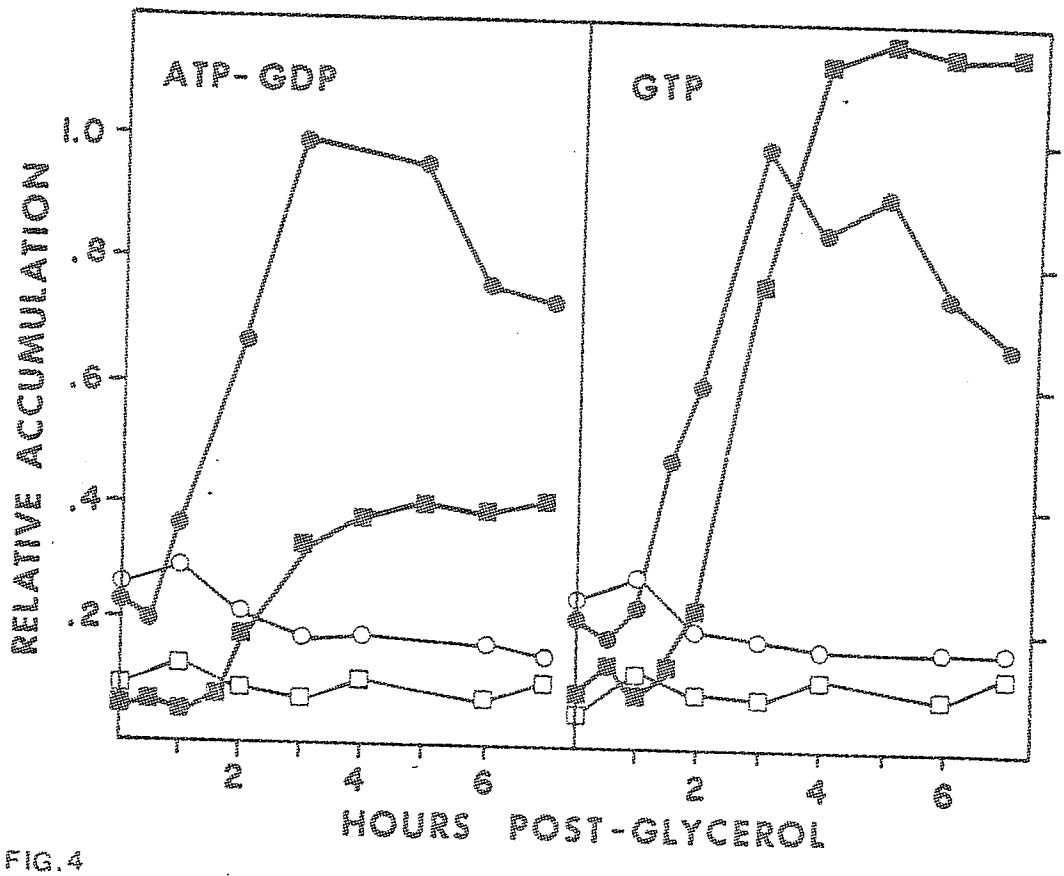

ATP-GDP
GTP
RELATIVE ACCUMULATION
1.0
.8
.6
.4
.2
2
4
6
2
4
6
HOURS POST-GLYCEROL

FIG. 4

The ratio of label in supernatant to the cellular nucleotides was always higher for GTP than ATP-GDP.

The labelling of UTP and CTP followed a pattern similar to that of GTP and ATP-GTP, although increases were not as marked (Fig. 3 and Fig. 5). In this instance, the ratio of label in the supernatant to cellular nucleotides was about the same. Noticeably, there was a far greater amount of label present in cell extracts than in supernatant fractions of sporulating cells; both being greater than the controls.

Glycerol induction was observed to have similar effects on some of the deoxyribonucleotides, dNTP's. Figure 6 shows the accumulation patterns of the dNTP's observed. In order to resolve the dNTP's that ran near the solvent front, it was necessary to change the molar strength of the phosphate buffer to 1.0 M KH_2PO_4, pH 3.65. Of the dNTP's examined, only TTP could not be detected supernatant fractions. While both TTP and dATP accumulation was similar to the ribonucleotides, that of dGTP and dCTP exhibited reverse patterns. That is, in both cases, the extracts of vegetative cells showed higher levels of these compounds than that of induced cells. Similarily, the supernatant fractions from vegetative cells contained the same or greator levels than that of induced cells.

These findings demonstrated that glycerol inductions influenced overall nucleotide metabolism. It should be noted that in control cultures the radioactivity in the nucleotides, calculated on a cell basis, remained constant from time zero

Fig. 5. Accumulation of ^{32}Pi (250 uCi/ml) into CTP and UTP by glycerol-induced cultures (closed symbols) and control, vegetative cultures (open symbols). Glycerol or water was added at zero time, 30 min after addition of ^{32}Pi, and cell extracts (●,○) and cultures supernatant extracts (■,□) were analyzed (Methods). The counts shown are relative to the maximum amount incorporated into cell extracts by induced cultures. For CTP the maximum was 45,000 cpm/10^8 cells and for UTP it was 37,000 cpm/10^8 cells.

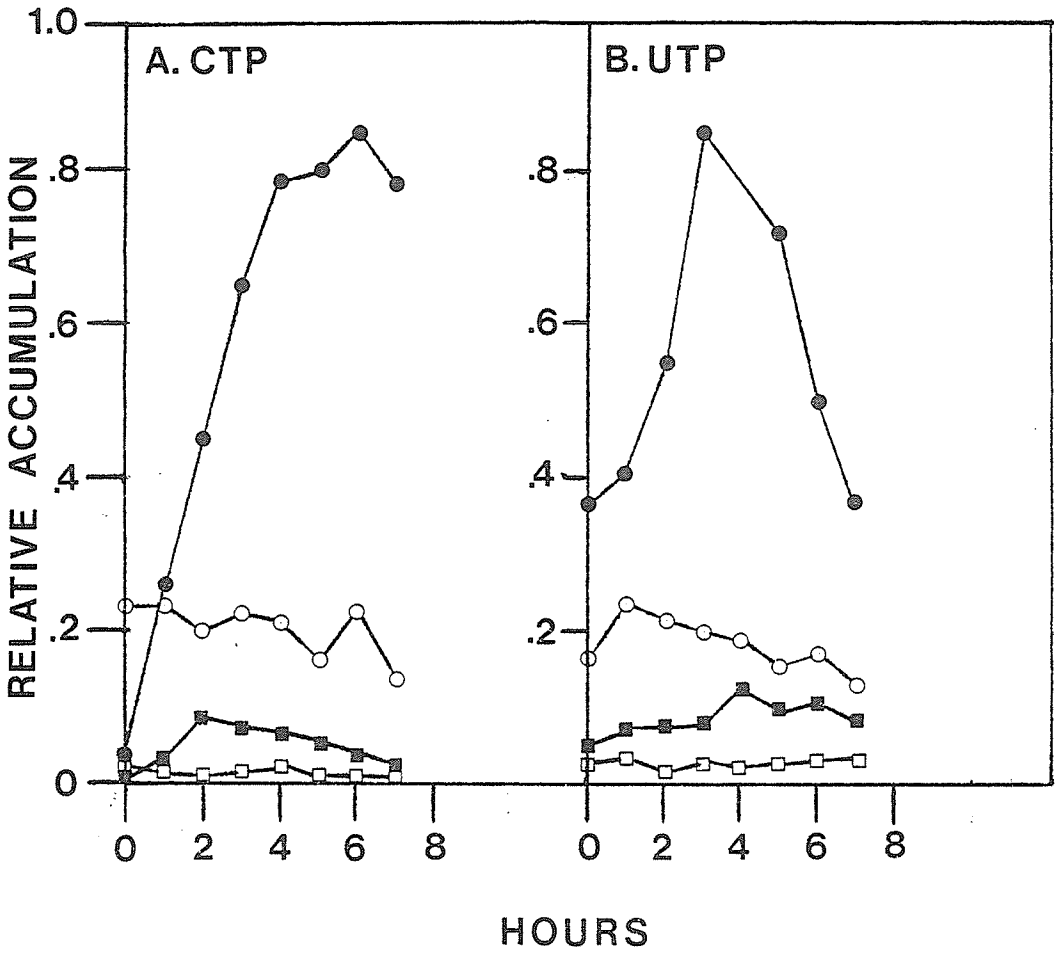


FIG.5

(Fig. 3). This indicated that the 30 min pre-labelling time, prior to glycerol addition was sufficient for equilibration of the label and that accumulation was not a result of increasing specific activities in phosphate pools. Accumulation of ^{32}Pi into nucleotides was basically the same regardless of pre-labelling periods up to 4 h.

To determine the amount of ATP present in the cellular pool, the firefly luciferase method described by Strehler and Totter (1954) was employed. Control and glycerol-induced cultures were grown as in previous experiments except ^{32}Pi was not added and 1ml samples were collected at various times, filtered then assayed as described in Methods.

In induced cultures, a 2-fold increase in ATP levels was observed, but this remained constant in control cultures (Fig. 7). This smaller increase of ATP as compared to that observed when ^{32}Pi was used to label ATP may have resulted from differences in the extraction procedures (see Methods). It may also indicate that the ATP formed during glycerol induction proceeded by de novo and/or salvage pathways whereby more than 1 phosphate molecule was incorporated. At any rate, the accumulation of the label into ATP, and presumably, other nucleotides, was due in part, to increasing pool sizes.

To determine whether increased rates of synthesis of nucleotides was responsible for accumulation, pulse label studies were carried out. Control and glycerol-induced cultures were pulsed for 30 min with ^{32}Pi over a 5 h period. After extraction, the amount of label incorporated into the

Fig. 6. Incorporation of ^{32}Pi (250 uCi/ml) into the various dNTP's, by glycerol-induced cultures (open symbols) and vegetative control cultures (closed symbols). Glycerol or water was added at zero time, 30 min after the addition of ^{32}Pi, and cell extracts (●,○) and supernatant extracts (■,□) were analyzed (Methods). Counts shown are actual to that incorporated at the corresponding time.

FIG.6

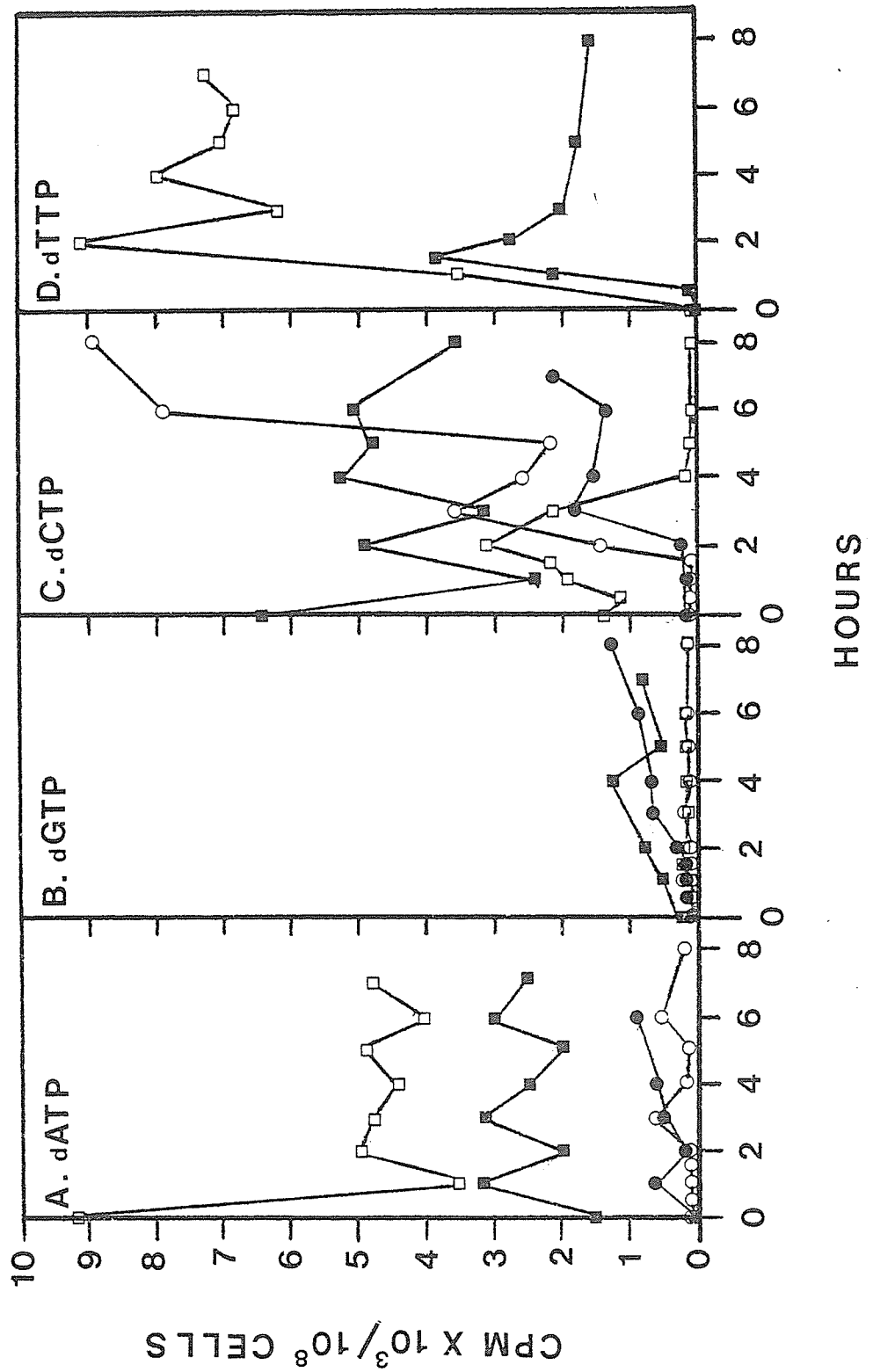

A. dATP
B. dGTP
C. dCTP
D. dTTP
CPM X $10^3/10^8$ CELLS
HOURS
0
1
2
3
4
5
6
7
8
9
10
0
2
4
6
8

Fig. 7. ATP levels in glycerol-induced cultures (●) and control vegetative cultures (○). Samples taken at times indicated were extracted in boiling water, and ATP content was measured by the firefly luciferase assay system described in the text.

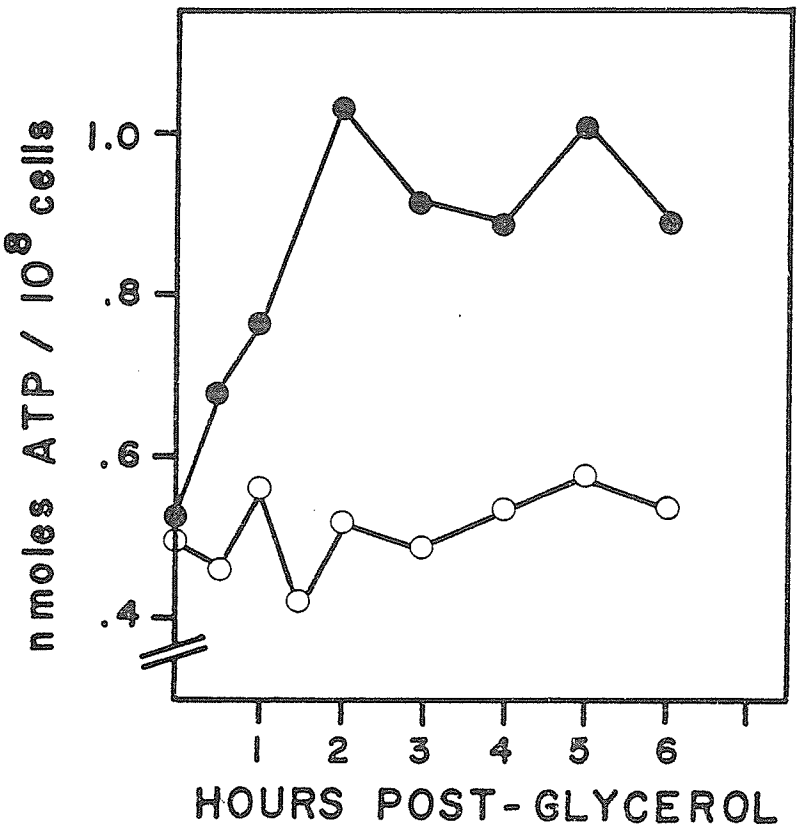


FIG. 7

Fig. 8. Pulse-labeling of cell extract GTP with ^{32}Pi (250 uCi/ml) during myxospore formation. Glycerol-induced cultures (1.0ml) were incubated for 30-min intervals with ^{32}Pi at times indicated, then centrifuged, extracted, and analyzed (see the text). The first vertical bar represents incorporation by vegetative cells that were pulsed 1 h after transfer to fresh medium.

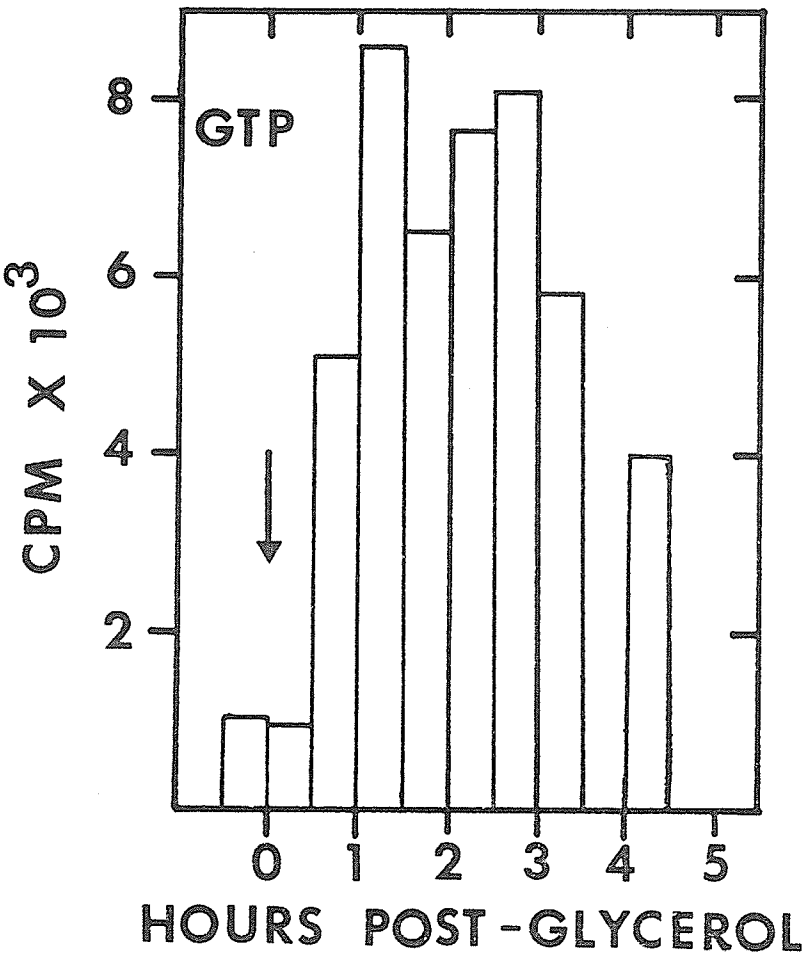


FIG. 8

nucleotides was determined as described in Methods.

As illustrated in Figure 8, the amount of ^{32}Pi incorporated into intracellular GTP increased 5-8 fold during myxospore formation as compared to vegetative cells. In Figure 8, the first vertical bar represents the amount of label incorporated by vegetative control cultures. Increased incorporation commenced 30 min after induction, and proceeded at maxinal rates for 2 h before decreasing. The fact that significant amounts of GTP were synthesized at 4 h post-induction when accumulation has ceased and may even be decreasing (Fig. 4) indicates that turnover may be taking place. Similar results (Fig. 9) were obtained when incorporation into ATP-GDP was determined.

Accumulation of ^{32}Pi into Compounds in Supernatant Extracts:

Although the above results do not give detailed information regarding the metabolism of nucleotides during myxospore formation, the data indicates that nucleotides accumulated during glycerol-induced myxospore formation. This accumulation may be important in explaining the appearances of the phosphorylated compounds A, B, C, D, E and O, that appear predominantly in the culture supernatant fluids during myxospore formation (see later). One dimensional chromotography was employed to analyze the incorporation of ^{32}Pi into these compounds in supernatant extracts. Spots G4P (ppGpp), 2 and 3 (Fig. 3) were not present in the supernatants and therefore did not interfere with the analysis. Figure 10 depicts the

Fig. 9. Pulse-labeling of cell extract ATP-GDP with ^{32}Pi (250 uCi/ml) during myxospore formation. Glycerol-induced cultures (1.0 ml) were incubated for 30-min intervals with ^{32}Pi at times indicated, then centrifuged, extracted, and analyzed (Methods). The first vertical bar represents incorporation by vegetative cells that were pulsed 1 h after transfer to fresh medium.

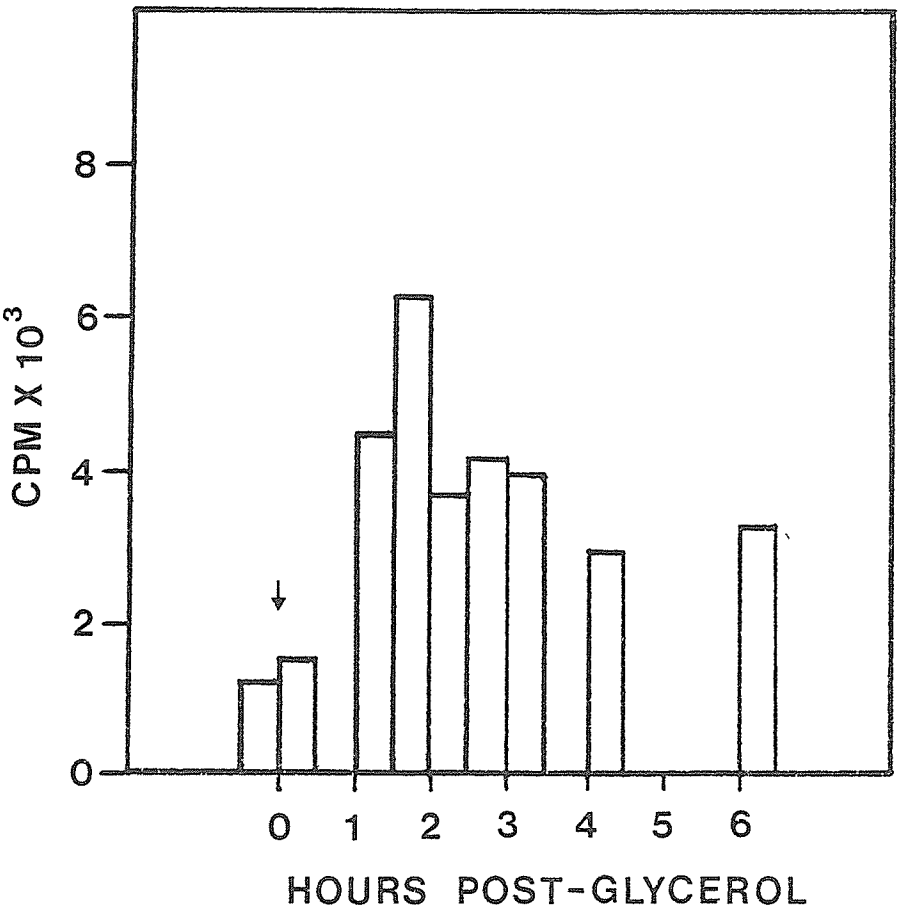
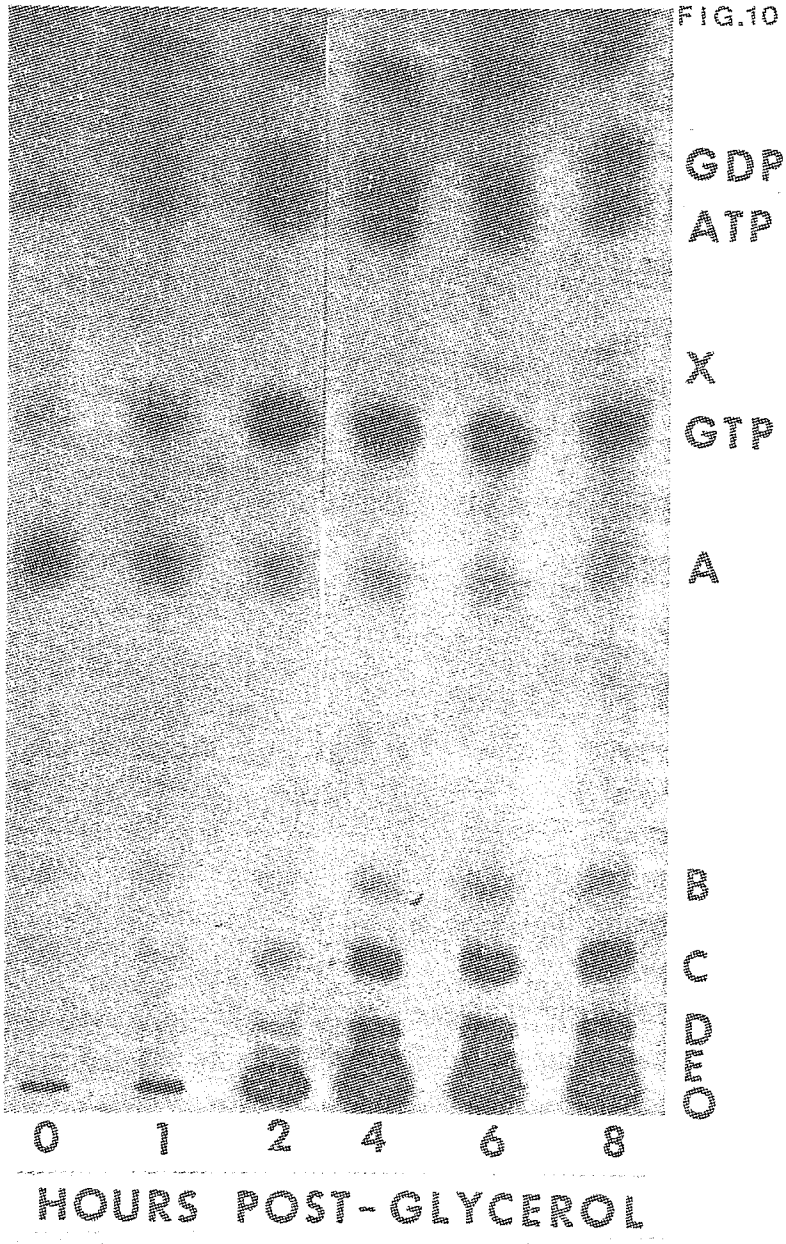


FIG.9

Fig.10. One-dimensional chromatography of supernatant extracts from glycerol-induced cultures. ^{32}P-labeled cultures (250 uCi/ml) were induced at zero time, and samples were removed at times indicated. A 5ul sample of the extract was run on PEI-cellulose thin-layer plates (20 by 40 cm). Only the lower 20 cm of the chromatogram is shown.

FIG.10



time course of appearance of labelled supernatant compounds during myxospore formation. After 30 min labelling of vegetative cells (Fig. 10, time 0), incorporation into ATP, GTP, spot A, spot O, and traces into B and C were observed. After glycerol induction, the intensity of labelling increased in all compounds with the exception of spot A, in which it appeared to decrease to a constant level. Extracellular nucleotides began accumulating between 1 and 2 h post-induction, whereas label did not accumulate into the series of spots B through O until 2 to 4 h after induction. Spot X, just above GTP did not appear until 6 h after glycerol addition.

The accumulation of label into spot O was followed in sporulating and vegetative cultures. During myxospore formation, there was a 50-fold increase in labelling of this material in supernatant extracts (Fig. 11A), reaching a plateau 4 h after induction. In contrast, ^{32}Pi did not accumulate into intracellular compound O, and the low levels present declined still further between 3 and 4 h post-induction (Fig. 11B) suggesting the material was excreted from sporulating cells. In control cultures, compound O accumulated slowly in both cell and supernatant extracts, but the total amount labelled after 6 h was 20-fold less than in the sporulating cultures (Fig. 11B).

The accumulation of ^{32}Pi into spot C is illustrated in Fig. 12. The incorporation patterns into spots D and E were almost identical to that of spot C and are not shown. Accumulation into supernatant and cell extracts began 2 h after

glycerol addition and continued to 7 h post-induction. Most, but not all, of compound C accumulated in the supernatant extracts. In the vegetative culture supernatant extracts, there were detectable amounts of C at zero time which remained constant, so that the amount per cell decreased with time (Fig. 12) suggesting that the cells did not synthesize this material. It was not detected in cell extracts from labelled vegetative cultures. Although the radioactivity accumulating in spot B was smaller, the pattern of accumulation was the same as that for spot C (Fig. 12).

The amount of label incorporated, in 30 min pulses, into the supernatant compounds was measured and is shown, in Fig. 13, for some of the compounds. In all cases, maximum incorporation occurred between 1 and 3 h post-induction. The increase in the rate of incorporation during myxospore formation can be judged by comparison with that incorporated by control cultures, as depicted by the first bar in each of the histograms (Fig. 13). The data indicated that the increased accumulation noted in Fig. 11 and 12 was due in part to increased rates of synthesis. On occasion, anomalous pulse-labelling was noted, as shown for spot C in Fig. 13, where incorporation rates were much higher than expected although the amount of increase was not. This was noted especially for supernatant compounds with greater chromatographic mobilities, i.e., spots A, B and C. The results appeared as artifacts due to high background levels of the compound. Subsequently, it was found that the background

Fig.11. Accumulation of ^{32}Pi into spot O in glycerol-induced cultures (closed symbols) and control vegetative cultures (open symbols). (A) Accumulation in supernatant extracts from cultures engaged in myxospore formation. (B) Accumulation in cell extracts of sporulating cultures (■) and vegetative cultures (□) and in supernatant extracts of vegetative cultures (O). Glycerol or water added at zero time, 30 min after addition of ^{32}Pi (250 uCi/ml).

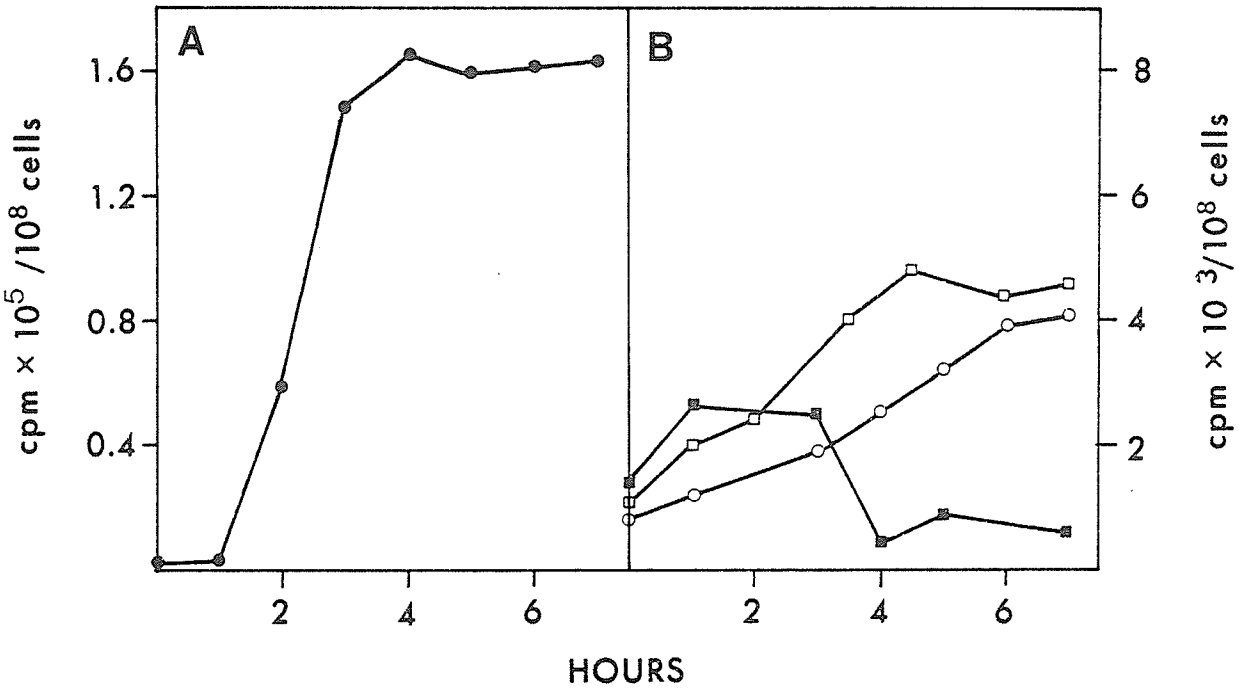

A
B
1.6
1.2
0.8
0.4
8
6
4
2
2
4
6
2
4
6
HOURS
cpm × 10^5 /10^8 cells
cpm × 10^3/10^8 cells

FIG. 11

Fig.12. Accumulation of ^{32}Pi (250 uCi/ml) into spot C in cell extracts (●) and supernatant extracts (O) from glycerol-induced cultures and into supernatant extracts (□) of control vegetative cultures. Also shown is the labeling of spot B (Δ) in supernatant extracts from sporulating cultures. Conditions for labeling are described in the legend to Fig. 11.

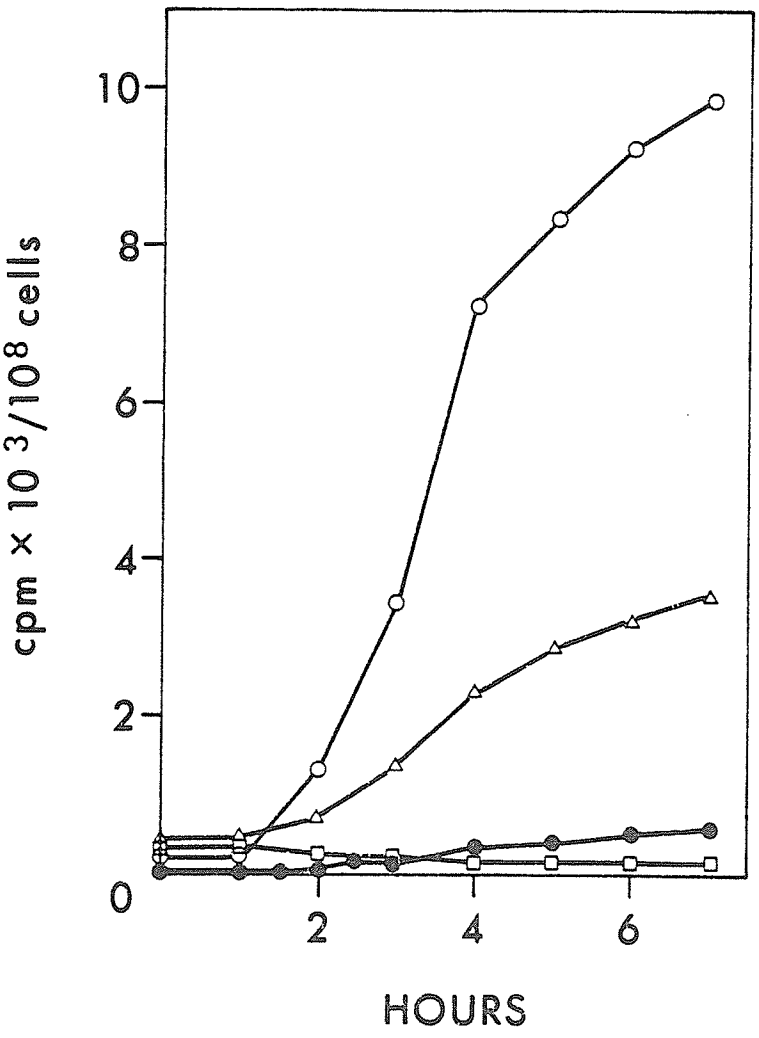


FIG.12

levels were due to contaminants present in the commercial ^{32}Pi stock.

Figure 14 illustrates an autoradiogram after chromotography of ^{32}Pi, taken directly from a commercial preparation, as compared to that of labelled supernatant extracts from cultures 8 h after induction. Although the bulk of the radioactivity in the commercial stock migrated as Pi, contaminants with the same mobilities as spots A, B, C, D and O were clearly visible. The presence of the contaminants explains the anomalous result observed in pulse label experiments (Fig. 13). Also, the appearance spots A, B, C, D and O at zero time in the accumulation experiments (Fig. 10 and 12) was likely due to these contaminants. Since the contaminants were likely to be inorganic polyphosphates (Brandhorst and Fromson, 1976), then the material that accumulated in culture fluids during myxospore formation was probably inorganic polyphosphates.

To show that sporulating cells did not selectively concentrate and release contaminants present in the label, accumulation of these compounds was measured with purified ^{32}Pi (see Methods). The purity can be judged by comparison of the Pi channels in Figures 14 and 15. Also shown in Figure 15 is the accumulation of the same compounds when purified ^{32}Pi was used for labelling. The amounts accumulated were basically the same as in the previous experiments except for the zero time counts which were lower for spots A and O (not shown). Portions of the chromatogram corresponding to positions

Fig.13. Pulse-labeling of spot O, spot D, and spot C in culture supernatant extracts during myxsopore formation. Glycerol-induced cultures (1.0 ml) were incubated for 30 min intervals with 250 uCi of ^{32}Pi at indicated times, and, after centrifugation, the supernatant was extracted and analyzed (see the text). The first bar in each of the histograms represents incorporation into control vegetative culture supernatants that were pulsed 1 h after transfer to fresh medium. The incorporation is given relative to the maximum incorporated into each spot, which was 18,600 cpm/10^8 cells for spot O, 400 cpm/10^8 cells for spot D, and 1,680 cpm/10^8 cells for spot C.

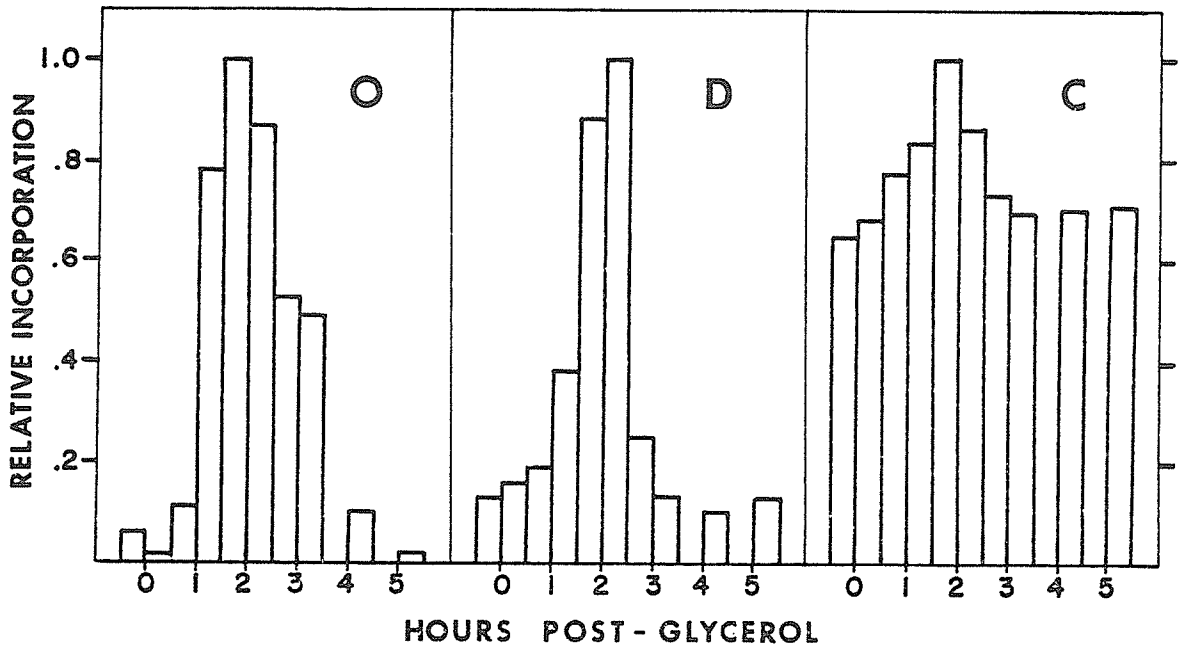

RELATIVE INCORPORATION
1.0
.8
.6
.4
.2
O
D
C
0 1 2 3 4 5
0 1 2 3 4 5
0 1 2 3 4 5
HOURS POST - GLYCEROL

FIG. 13

Fig.14. Comparison of ^{32}P-labeled supernatant extract from glycerol-induced cultures (left) and 10 uCi ^{32}Pi (right) by one-dimensional chromatography of PEI-cellulose thin-layer plates. The supernatant sample is the same as in Fig. 5. Both chromatograms were run under identical conditions, though not at the same time.

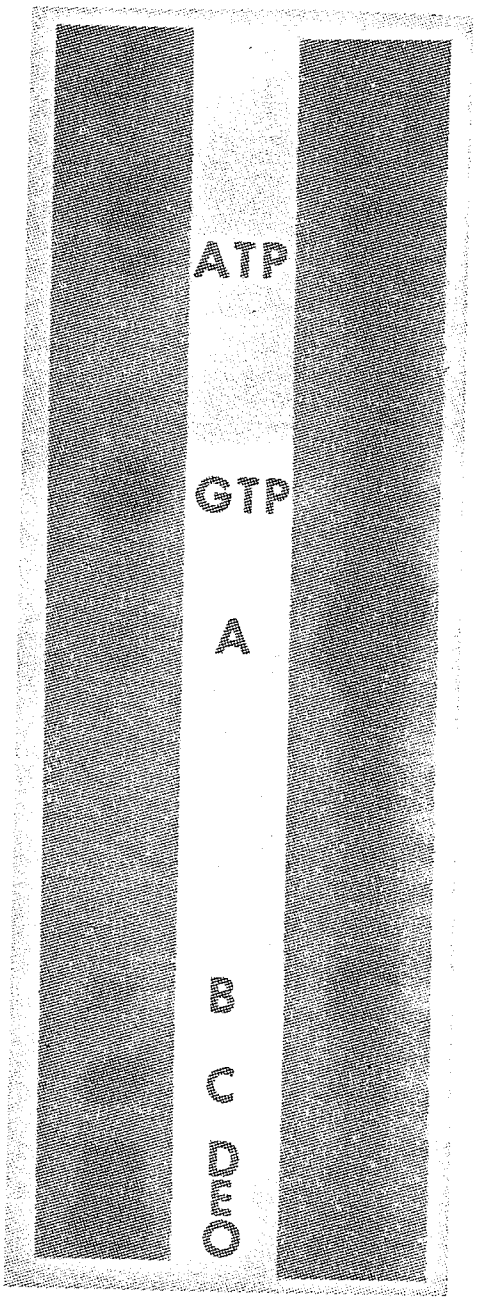


FIG.14

of traces of contaminants in the purified ^{32}Pi were cut out and counted. From the counts, it was estimated that if contaminants were responsible for the appearance of excreted material, their levels in the medium would have to be .5-to 10-fold greater to produce the results in Figure 15.

Further confirmation that the material accumulating in the medium was polyphosphates came from charcoal adsorption studies. Acid washed charcoal was mixed with ^{32}P-labelled supernatant extracts from cells induced for 4 h according to the procedure by Griffin et.al., (1965). After adsorption, only GTP, ATP and GDP could be detected in the eluates (channel 2, Fig. 16). The material that did not adsorb to charcoal (channel 3, Fig. 16) was identified as polyphosphates. When the unadsorbed material was incubated with inorganic pyrophosphatases, spots A and B were degraded (channel 4, Fig. 16), indicating that these may be pyrophosphate and possibly tripolyphosphate respectively. Treatment with alkaline phosphatase, organic pyrophosphates, ribonuclease A, spleen and snake venom phosphodiesterase and various combinations of these exzymes did not alter the mobilities of the unadsorbed compounds.

Labelling of Glycerol Non-Inducible Mutants:

The labelling of nucleosides and other phosphorylated compounds was analyzed in five independantly isolated mutants of M. xanthus that were unable to form myxospores in 0.5 M glycerol. The pattern of labelling after addition of glycerol

Fig.15. One-dimensional chromatography of supernatant extracts from glycerol-induced cultures labeled with ^{32}Pi (200 uCi/ml) purified as described in the text. Approximately 10 uCi of the purified label was chromatographed in the "Pi" channel. Samples were removed at indicated times, and the supernatant, after extraction, was chromatographed with 1.65 M phosphate buffer (pH 3.65).

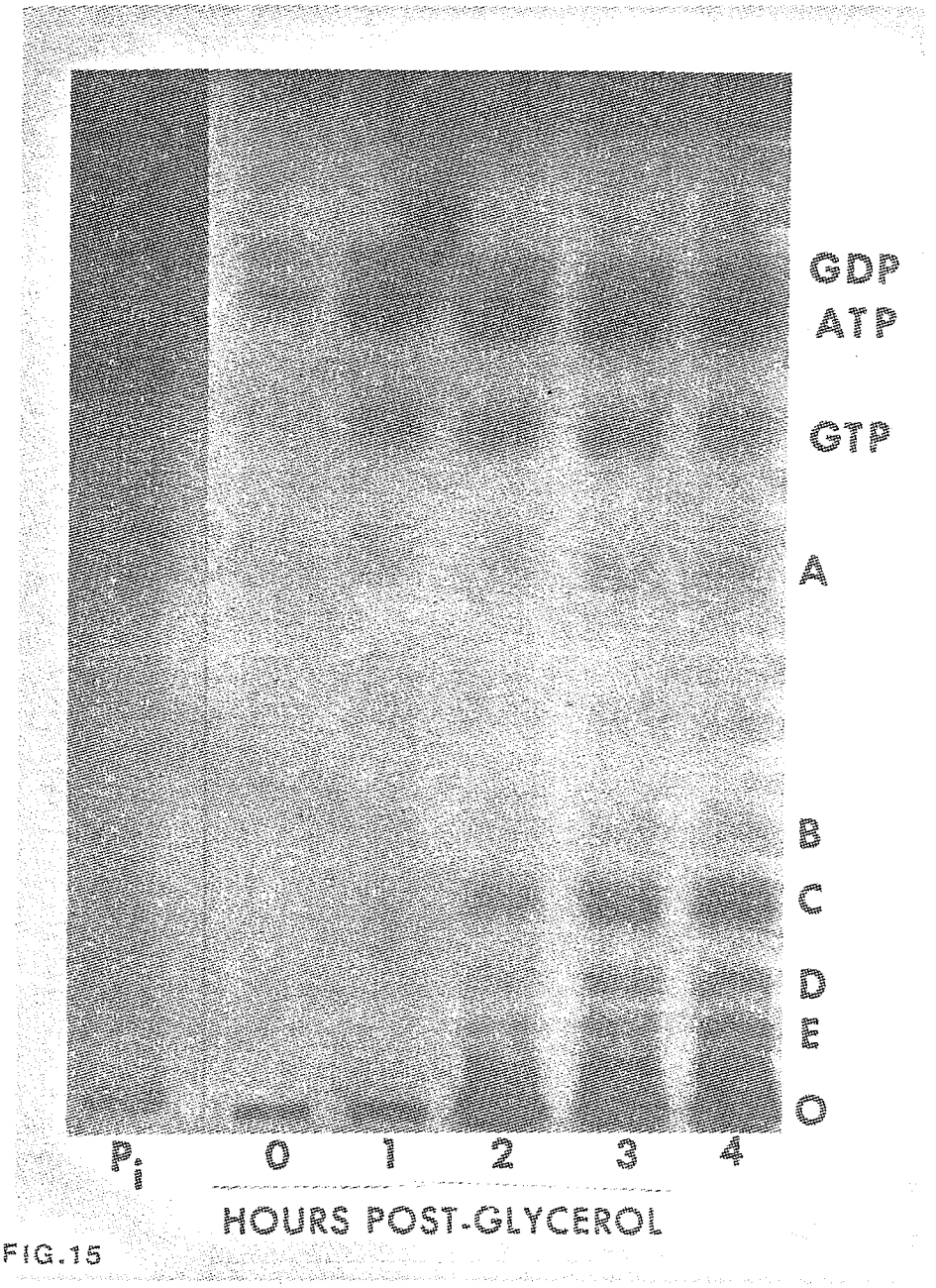


FIG. 15

Fig.16. One-dimensional chromatography of ^{32}P-labeled supernatant extracts fractionated by charcoal (see the text). Extracts were from cultures labeled for 4 h after glycerol induction. Channel 1 shows extracts before charcoal adsorption. Channel 2 shows the material eluted from charcoal, after adsorption, by water-ethanol-NH_3 (65:35:3). The remaining channels show the material that did not adsorb to charcoal before (3) and after (4) incubation with 100 ug of inorganic pyrophosphatase per ml for 30 min at 30°C.

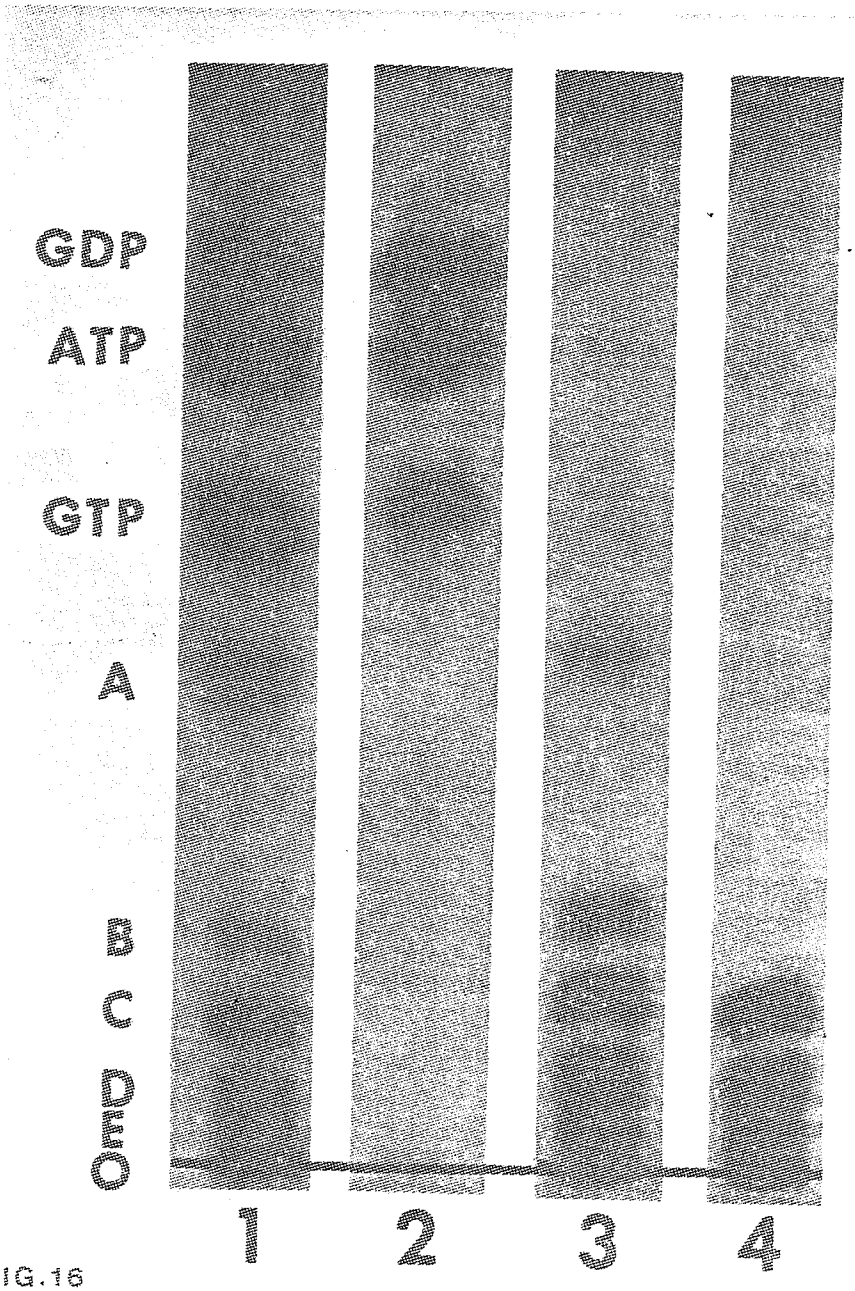


FIG. 16

was the same as that of vegetative cells in the absence of glycerol. The radioactivities of intracellular compounds remained constant (not shown), and those of some extracellular compounds are shown in Fig. 15. The radioactivity of GTP remained constant, and that of spot O increased as in vegetative cells (see above). The levels of polyphosphates A, B, C and D declined (Fig. 17); this was probably due to the presence of contamination compounds in the stock ^{32}Pi which were degraded and/or diluted as the cells grew. At any rate, the increased labelling of phosphorylated compounds observed during glycerol induced sporulation was not due to glycerol per se, but accompanied the formation of myxospores.

The Involvement of ppGpp

Co-chromatography with standards tentatively indentified one of the spots running beneath and slightly to the left of GTP as guanosine tetraphosphate (ppGpp, Fig. 3). This compound could not be detected in any of the supernatant extracts analyzed. However, the cell extracts examined contained detectable amounts of ppGpp.

To determine the level of ppGpp in vegetative cells, cells were cultured in 1% casitone (Difco), centrifuged and resuspended in fresh medium. After 30 min, ^{32}Pi was added (time 0) and samples taken at various times. Samples were then extracted as described in Methods and analyzed for ppGpp. Cell extracts revealed only negligible amounts of ppGpp (Fig. 18). To determine the effect of nutrient

Fig.17. Accumulation of ^{32}Pi into cultures supernatant extracts by a glycerol-noninducible mutant growing in 0.5 M glycerol and 250 uCi of ^{32}Pi per ml .

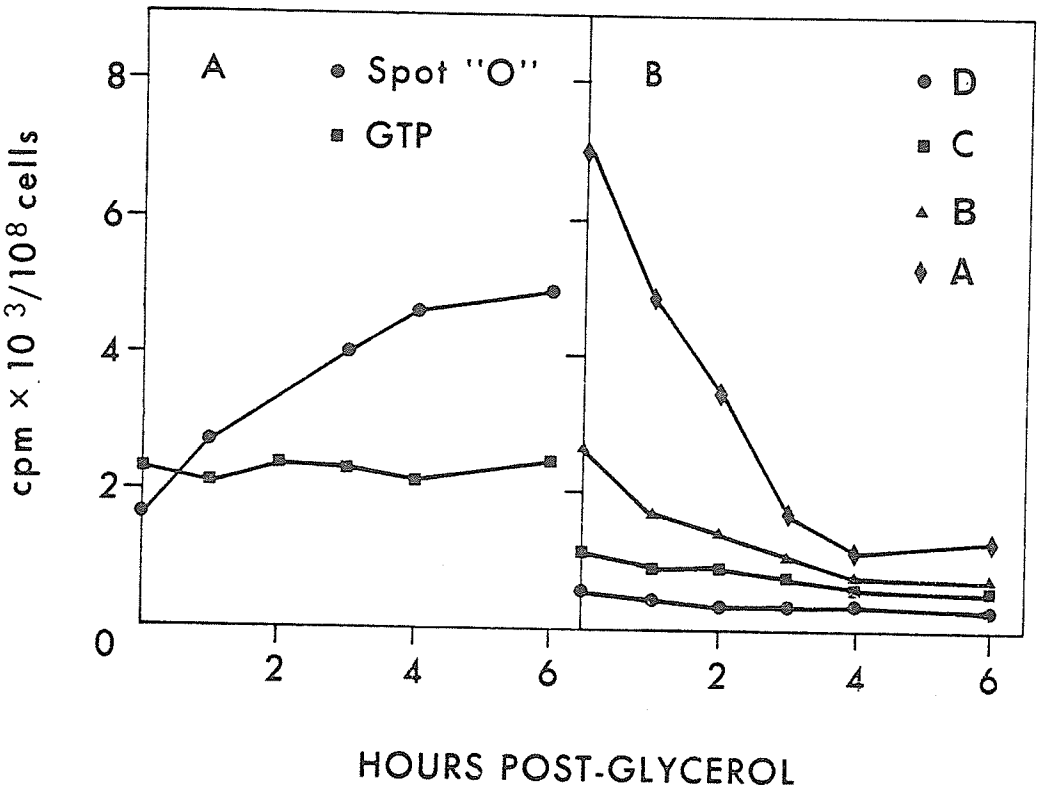

A
Spot "O"
GTP
B
D
C
B
A
8
6
4
2
0
2
4
6
2
4
6
cpm × 10 3/10 8 cells
HOURS POST-GLYCEROL

FIG. 17

concentration on ppGpp accumulation, cells grown in 1% casitone were transferred to 0.1% casitone and allowed to grow vegetatively for 30 min prior to the addition of ^{32}Pi (time 0). As before samples were taken at various times, extracted and analyzed for ppGpp (see Methods). Under these conditions, the levels of ppGpp were significantly greater, suggesting that ppGpp accumulation may be controlled by a "stringent response" similar to that seen in E. coli (Cashel and Gallant, 1969).

The accumulation of ppGpp was next examined in sporulating cells. As above cells were resuspended in 1.0% and 0.1% casitone and allowed to grow 30 min prior to the addition of ^{32}Pi and glycerol (0.5 M final concentration) which signified time 0. As before samples were removed and analyzed for ppGpp as described in Methods (Fig. 19). It was observed that the levels of ppGpp from sporulating cells cultured in 0.1% casitone was far greater than the level observed from sporulating cells cultured in 1.0% casitone. Whereas the levels of ppGpp decreased throughout the induction period in 0.1% casitone, a slight increase was noticeable at 2 h in 1% casitone. Unlike the cells grown in 1.0% casitone which converted wholely to spores, the cells raised in 0.1% casitone underwent only a 5% conversion to spores.

To determine whether the slight increase of ppGpp observed during induction in 1.0% casitone was real, the experiment was repeated. The pattern of ^{32}Pi-incorporation into ppGpp of cell extracts from glycerol-induced cultures

Fig.18. Incorporation of ^{32}Pi (100 uCi/ml) into ppGpp, Guanosine tetraphosphate in cell extracts of vegetative cells grown in 1% casitone (■) and in 0.1% casitone (●).

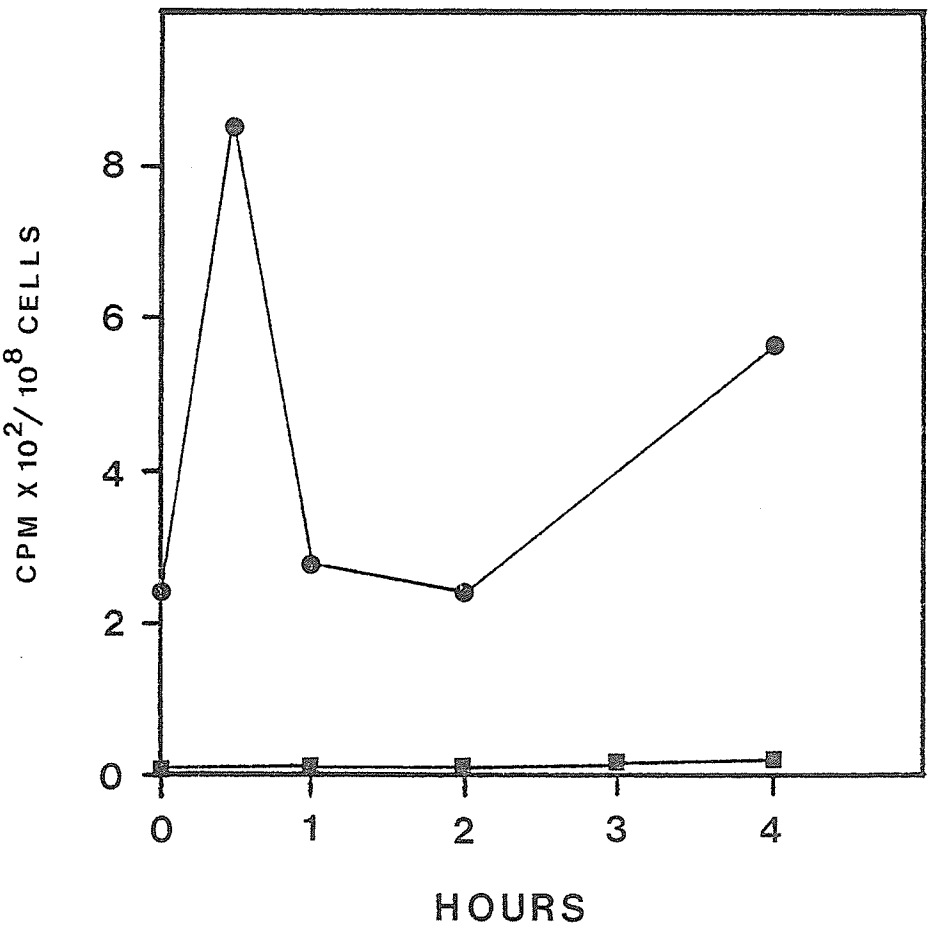


FIG. 18

grown in 1% casitone is illustrated in Figure 20. After the introduction of glycerol (i.e. initiation of sporulation), maximum accumulation occurs between 2 and 3 h after induction. This correlates well with the times at which reduction in net RNA synthesis takes place (K. Bacon and E. Rosengerg, 1967; W.S. Ramsay and M. Dworkin, 1970). Guanosine pentaphosphate was not detected under any of the conditions examined, however it should appear between spots B and C in Fig. 3.

Other Phosphorylated Compounds

As previously mentioned, spots 2 and 3 are two more compounds not seen in the supernatant extracts. While spot 3 was always present, analysis was not possible due to streaking and possible degradation within the chromatographic system employed. However spot 2, whose appearance is more predictable was not subject to this breakdown during chromarography. It would also appear that spot 2 is not subject to the influence of nutrient concentration, unlike ppGpp (Fig. 21). It is possible that spot 2 is related to HPN I which (Rhaese and Groscurth, 1974) accumulated in all extracts of stationary phase Bacillus cells.

Fig.19. Incorporation of ^{32}Pi (250 uCi/ml) into ppGpp in all extracts of induced cells grown in 1% casitone (▩) and 0.1% casitone (●). In 1% casitone there was 100% conversion to spores which became refractile in 4 h. In 0.1% casitone there was less than 5% conversion to spores.

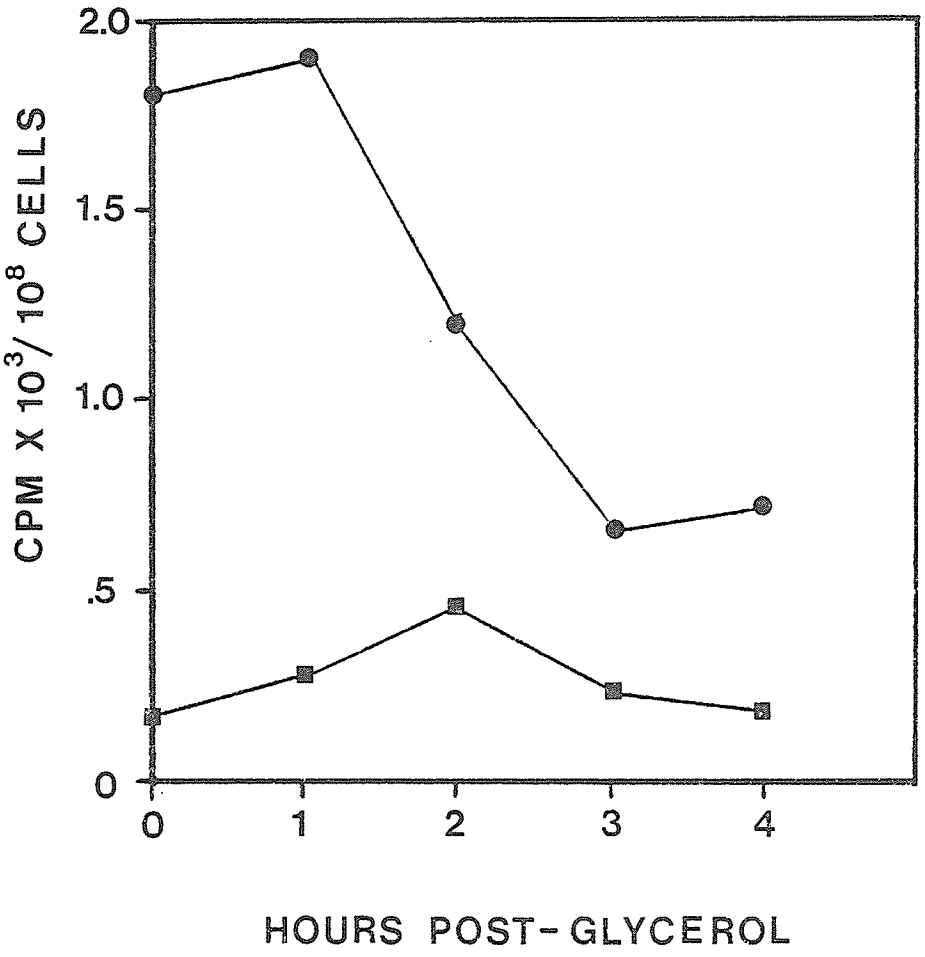


FIG. 19

Fig.20. Incorporation of ^{32}Pi (250 uCi/ml) into ppGpp in cell extracts of glycerol induced cultures. Glycerol was added at zero time, 30 min after the addition of the label, and cell extracts were prepared at indicated times

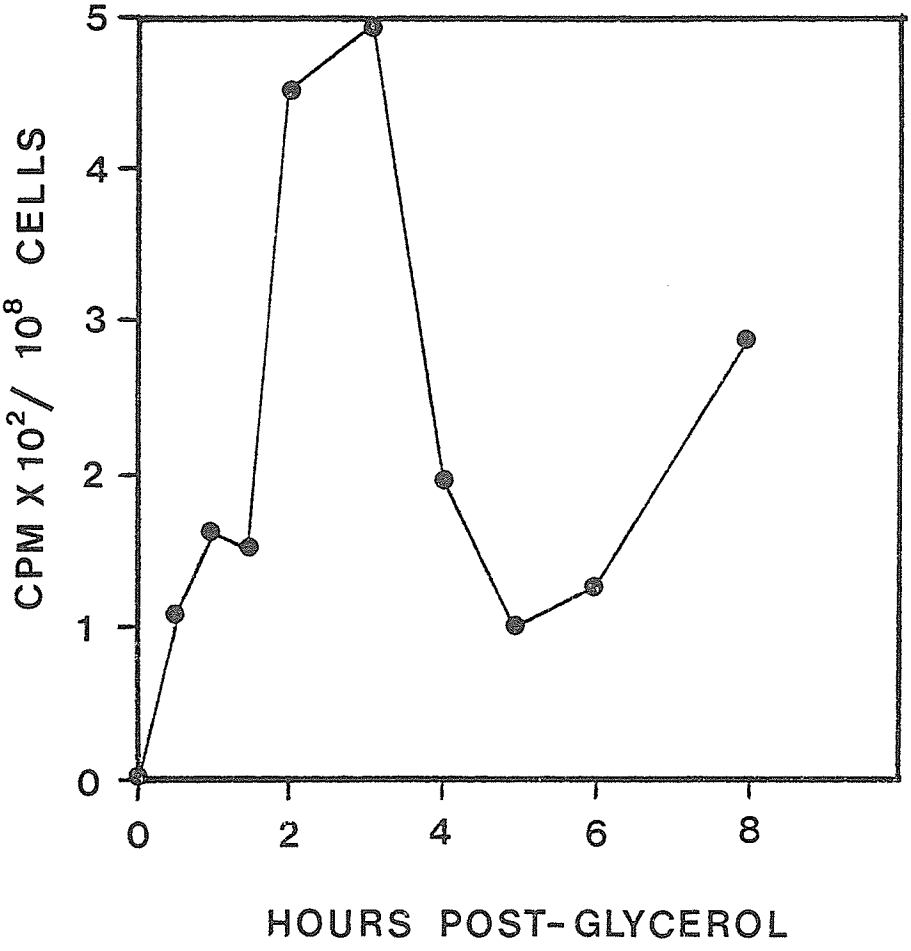


FIG. 20

Fig.21. Incorporation of ^{32}Pi (250 uCi/ml) into spot 2 in glycerol-induced extracts of cells grown in 1% casitone (●) and cells grown in 0.1% casitone(○).

FIG. 21

Fig.22. Incorporation of ^{32}Pi (250 uCi/ml) into spot 2 in stationary phase cells grown in 1% casitone.

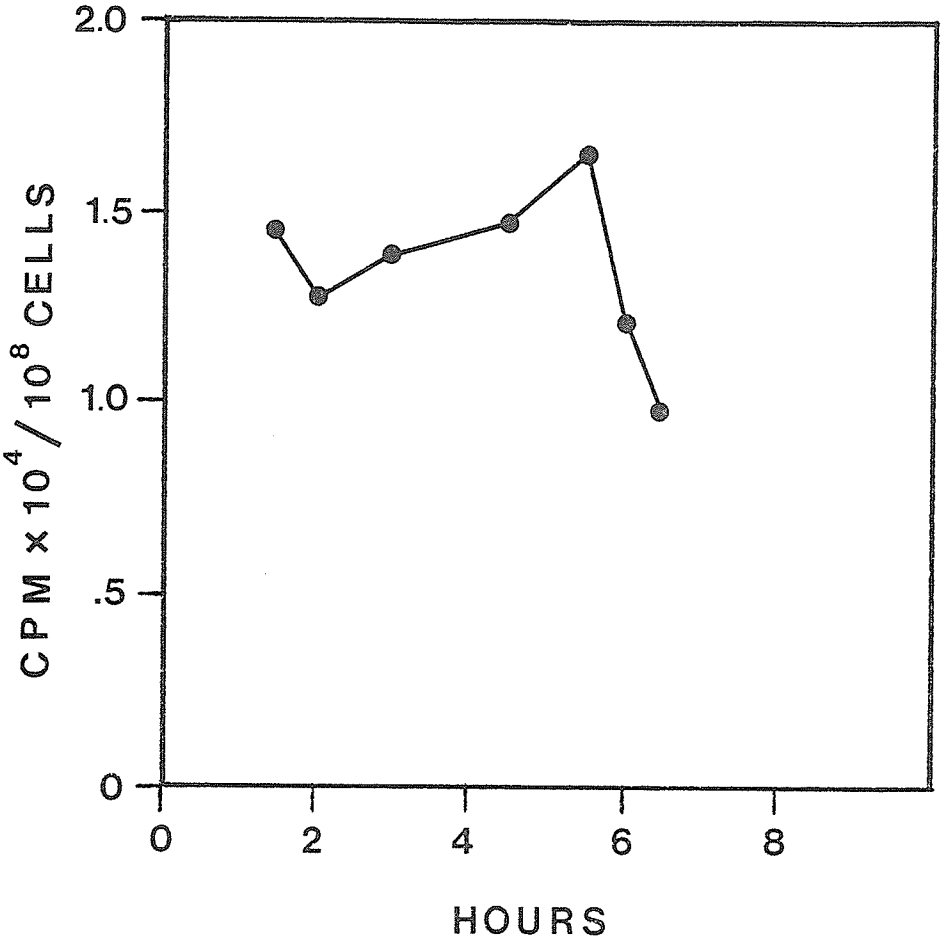


FIG. 22

DISCUSSION

During myxospore formation, the amount of ^{32}Pi incorporated into nucleotides was approximately fivefold greater than in vegetative cells. Although the ratio of intracellular/extracellular amounts varied, a significant portion was excreted into the medium of sporulating cultures. The effect was not restricted to nucleoside triphosphates, since in cases where ATP and GDP were resolved (as in Fig. 5) increased labelling into both compounds was observed. The accumulation could not be explained by RNA degradation that takes place during induction, since the turnover rate maintains RNA at a constant level (Bacon and Rosenberg, 1967; Okano et.al., 1970; Pao et.al., 1977). Nor could the effects be attributed to osmotic effects, since mutants that were not induced by glycerol failed to accumulate nucleotides, as well as other phosphorylated compounds, in the presence of glycerol. The fact that prelabelling periods up to 4 h yielded essentially the same results, indicates accumulation was not a result of increasing specific activities of phosphate pools. Futhermore, direct assays of ATP content of cells (Fig. 3) and pulse-label experiments (Fig. 4) indicated that the pool sizes and synthesis of nucleotides increased during glycerol-induced myxospore formation.

The period of maximum accumulation of nucleotides was one of active synthesis of spore-specific enzymes and spore material (Dworkin and Gibson, 1964; Filer et.al., 1977; Sutherland, 1976; White, 1975) and may represent a time of increased

production of nucleotides for energy and formation of spore-specific RNA. However, the marked accumulation of both intra- and extracellular nucleotides indicated an overproduction of these compounds for which there is no obvious explanation. Perhaps they represent nucleotide reserves in myxospores that may also be required for germination. In Bacillus species, increases were noted in nucleotide pools during sporulation (Chow and Takahashi, 1972; Singh et.al., 1977).

Their accumulation predominantly in culture supernatant and the kinetics of accumulation and synthesis indicated that spots O, B, C, D and E were related compounds that were likely polyphosphates of varing chain lengths (see Results). Recently, Ludwig et.al., (1977) showed that polyphosphates could be identified by the chromatographic procedure of Cashel (1969) as used in this study. Comparison of the data shows that spots A, B, and C were likely pyrophosphate and tri- and tetra-phosphates, respectively. The other compounds, D, E, and O, are most likely polyphosphates with greater chain lengths. Since spot O did not migrate in this system, it was not possible to determine whether it was comprised of one or more compounds. If these spots represent a family of polyphosphates, then spot O would be the largest and may be the parent molecule from which the others were formed. It reached its peak level within 3 h after glycerol addition (Fig. 6), whereas the others showed greatest accumulation between 3 and 4 h and continued to increase (Fig. 7). Also spot O accumulated in vegetative

cells and showed the greatest increase during myxospore formation. The presence of contaminants in stock ^{32}Pi, presumably polyphosphate (Brandhorst and Fromson, 1976), could not account for their appearance in the medium of sporulating cells (Results). Although the polyphosphates accumulated in much greater quantities in the medium, their presence in small amounts in cell extracts indicated that they were formed within the cytoplasm. During vegetative growth in 10 mM phosphate, polyphosphates were deposited as granules within cells (Voelz et.al., 1966). This is in contrast to the extracellular location of the polyphosphate species formed during myxospore formation; nevertheless, it does demonstrate that these organisms have the capacity to synthesize these polymers.

The accumulation of polyphosphates has been studied in other procaryotes. In Nitrosomonas, polyphosphate accumulated when generated ATP was not efficiently used to promote an increase in cell mass (Terry and Hooper, 1970). In Aerobacter, polyphosphate accumulated whenever nucleic acid synthesis ceased due to nutritional deficiency, regardless of its nature (Harold and Sylvan, 1963). During myxospore formation, net RNA synthesis is reduced after glycerol addition (Bacon and Rosenberg, 1967; Ramsay and Dworkin, 1970), at a time when nucleotides are accumulating (see Results). It is probable that under these conditions a diversion of ATP pools to polyphosphate takes place. The time at which polyphosphate synthesis was observed indicates polyphosphates do not play

a role in initiating myxospore formation. That guanosine tetraphosphate accumulated between 120 and 180 min (Fig. 13) suggests it may play a role in mediating the rates of RNA synthesis.

It seems that extracellular polyphosphates could serve as energy or phosphate reserves (Shively, 1974). This may be a mechanism by which internal phosphate levels are lowered to facilitate myxospore formation. It is known that the formation of spores in Myxococcus is inhibited by orthophosphate (Bacon and Rosenberg, 1967); also, their accumulation may be related to the observation that a germination factor that could be replaced by Pi was produced by germinating myxospores (Ramsay and Dworkin, 1970). Perhaps the polyphosphates, which were synthesized late during sporulation, accumulate and, with the onset of germination, are mobilized, e.g., by a polyphosphatase, to an active form that stimulates germination. Since the organism normally grows on solid medium where diffusion of polyphosphates may be limited, this scheme may not be far-fetched. Recent reports implicate polyphosphates in various regulatory functions (Hildebrandt and Sauer, 1977; Ludwig et.al., 1977).

The identity of spots 2, 3 and X remains unknown at this time. It does not appear likely that compound X plays a role in initiating myxospore formation since it appears fairly late in the process. Spots 2 and 3 accumulate from the onset of spore formation but data regarding their function and identity is elusive. It is interesting to note that spot 2 has chro-

matographic properties similar to HPN III reported by Rhaese et.al., (1975) in sporulating Bacillus. The reported structure is ppZpUp, where Z is a monosaccharide (Rhaese et.al., 1976). A role for this molecule in sporulation has not been determined.

Bibliography

Dworkin, M. 1966, Biology of the Myxobacteria.
Ann. Rev. Microbiol., 20, pp. 75-106

Bergey's Manual of Determinative Bacteriology, 8th Ed. 1974, pp 1246
R.E. Buchanan and N.E. Gibbons, Co. Eds.
Williams and Wilkins Co., Baltimore

Voelz, H. and M. Dworkin. 1962, Fine structure of Myxococcus xanthus during morphogenesis.
J. Bacteriol., 84:943-952.

White, D., M. Dworkin and J.D. Tipper. 1968, Peptidoglycan of Myxococcus xanthus: Structure and relation to morphogenesis.
J. Bacteriol., 95:2186-2197.

Dworkin, M. 1962, Nutritional requirements for vegetative growth of Myxococcus xanthus.
J. Bacteriol., 84:250-257.

Hemphill, H.E. and S.A. Zahler. 1968, Nutrition of Myxococcus xanthus FBa and some of its auxotrophic mutants.
J. Bacteriol., 95:1011-1017.

Hemphill, H.E. and S.A. Zahler. 1968, Nutritional induction and suppression of fruiting in Myxococcus xanthus FBa
J. Bacteriol., 95:1018-1023.

McCurdy, H.D. and B.T. Khouw. 1969, Studies on Stigmatella Brunnea.
Can. J. Microbiol., 15:731-738.

Dworkin, M. 1963, Nutritional regulation of morphogenesis in M. xanthus.
J. Bacteriol., 86:67-72.

Wireman, J.W. and M. Dworkin. 1975, Morphogenesis and developmental interactions in Myxobacteria.
Science, 189:516-522.

Burchard, R.P. and M. Dworkin. 1966a, Light-induced lysis and carotenogenesis in M. xanthus.
J. Bacteriol., 91:535-545.

Sudo, S.Z. and M. Dworkin. 1969, Resistance of vegetative cells and microcysts of M. xanthus. J. Bacteriol., 98:883-887.

Burchard, R.P. 1975, Myxospore development in a non-dispersed growing mutant of M. xanthus. Bacteriol. Proc., 75:132-306.

Burchard, R.P. 1974, Growth of surface colonies of the gliding bacterium M. xanthus. Arch. Microbiol., 96:247-254.

Dworkin, M. and S.M. Gibson. 1964, A system for studying microbial morphogenesis: Rapio formation of microcysts in M. xanthus. Science, 146:243-244.

Dworkin, M. and W. Sadler. 1966a, Induction of cellular morphogenesis in M. xanthus. I. General description.

_____ and _____. 1966b, Induction of cellular morphogenesis in M. xanthus. II. Macromolecelar synthesis and mechanism of inducer action. J. Bacteriol., 94:1516-1519, 1520-1525.

Rosenberg, E., M. Katarski and P. Gottlieb. 1967, DNA synthesis during exponential growth and microcyst formation in M. xanthus. J. Bacteriol., 93:1402-1408.

Bacon, K. and E. Rosenberg. 1967, RNA synthesis during morphogenesis in M. xanthus. J. Bacteriol., 94:1883-1889.

Orlowski, M. and D. White. 1974, Inactivation of isocitrate lyase during myxospore development in M. xanthus. J. Bacteriol., 118:96-102.

White, D. 1975, Myxospores of Myxococcus xanthus. Spores VI, P. Gerhardt, R.N. Costilow and H.L. Sadoff (ed.)., pp. 44-51. Amer. Soc. Microbiol., Washington, D.C.

Shimkets, L. and T.W. Seale. 1975, Fruiting-body formation and myxospore differentiation and germination in M. xanthus viewed by scanning electron microscopy. J. Bacteriol., 121:711-720.

Hodgkin, J. and D. Kaiser. 1977, Cell to cell stimulation of movement in non-motile mutants of Myxococcus. Proc. Nat. Acad. Sci., 74:2938-2942.

Watson, B.F. and M. Dworkin. 1968, Comparative intermediary metabolism of vegetative cells and microcysts of M. xanthus. J. Bacteriol., 96:1465-1473.

Orlowski, M.P., D. Martin, D. White and M. Wong. 1972, Changes in activity of glyoxlate cycle enzymes during myxospore development in M. xanthus. J. Bacteriol., 111:784-790.

Filer, D., S.H. Lindler and E. Rosenberg. 1977a & b, Myxospore coat synthesis in M. xanthus: Enzymes associated with uridine 5'-di-phosphate-n-acetylgalactosamine formation during myxospore development. J. Bacteriol., 131:745-788.

Witkins, S.S. and E. Rosenberg. 1970, Induction of morphogenesis by methionine starvation in M. xanthus. J. Bacteriol., 103:641-649.

Filer, D., E. Rosenberg and S.H. Kindler. 1973, Aspartokinase of M. xanthus: "Feedback stimulation" by required amino acids. J. Bacteriol., 115:23-28.

Campos, I.M. and D.R. Zusman. 1975, Regulation of development in M. xanthus: Effect of 3':5' cAMP, ADP and nutrition. Proc. Nat. Acad. Sci., 72:518-522.

Schwarte, D. and J.R. Beckwith. 1970, The Lactose Operon, pp. 411-422 J.R. Beckwith and D. Zipper (ed.) Cold Spring Harbor Laboratory Cold Spring Harbor, N.Y.

McVittie, A. and S.A. Zahler. 1962, Chemotaxis in Myxococcus. Nature (London), 194:1299-1300.

Ramsay, W.S. and M. Dworkin. 1968, Microcyst germination in M. xanthus. J. Bacteriol., 95:2249-2257.

Sands, M.K. and R.B. Roberts. 1952, The effects of tryptophan-histidine deficiency in a mutant of Escherichia coli. J. Bacteriol., 63:505-511.

Pardee, A. and L. Prestidge. 1956, The dependance of nucleic acid synthesis in the presence of amino acids in E. coli.
J. Bacteriol., 71:677-681.

Stent, G.S. and S. Brenner. 1961, A. genetic locus for the regulation of RNA synthesis.
Proc. Nat. Acad. Sci., 47:2005-2009.

Borek, E., J. Rockenbach and A. Ryan. 1956, Studies on a mutant of E. coli with unbalanced RNA synthesis.
J. Bacteriol., 71:318-325.

Cashel, M. and J. Gallant. 1969, Two compounds implicated in the function of the RC gene of E. coli.
Nature (London), 221:838-846.

Cashel, M. and B. Kalbacher. 1970, The control of RNA synthesis in E. coli: A characterization of a nucleotide associated with the stringent response.
J. Biol. Chem., 245:2309-2318.

Reiness, G., H. Yang, G. Zubay and M. Cashel. 1975, Effects of ppGpp on cell-free synthesis of E. coli rRNA and other gene products.
Proc. Nat. Acad. Sci., 72:2881-2885.

Nierlich, D.P. 1968, Amino acid control over RNA synthesis: A re-evaluation.
Proc. Nat. Acad. Sci., 60:1345-1352.

Lazzarini, R.A. and A.E. Dahlberg. 1971, The control of RNA synthesis during amino acid deprivation in E. coli.
J. Biol. Chem., 70:420-429.

Primakoff, P. and P. Berg. 1970, Cold Spring Harbor Symposium.
Quant. Biol., 35:391-396.

Ikemura, T. and A.E. Dahlberg. 1973, Small Ribonucleic acids of E. coli.
J. Biol, Chem., 248:5033-5041.

Klein, C. 1974, Presence of magic spot in Dictyostelium discoideum.
FEBS Let., 38:149-152.

Rhaese, H.J. 1975, Studies on the control of development synthesis of regulatory nucleotides, HPN and MS in mammalian cells in tissue culture.
FEBS Let., 53:113-118.

Irr, J.D., M.S. Kaulenas and B.R. Unsworth. 1974, Synthesis of ppGpp by mouse emeryonic ribosomes. Cell, 3:249-253.

Haseltine, W.A., R. Block, W. Gilbert and R. Weber. 1972, MS I and MS II made on ribosome in idling step of protein synthesis. Nature, 238:381-389.

Rhaese, H.J. and R. Groscurth. 1974, Studies on the control of development. In vitro synthesis of HPN and MS nucleotides by ribosomes from either sporulating or vegetative cells of Bacillus subtilis. FEBS Let., 44:87-93.

Gallant, J. and G. Margason. 1972, Amino acid control of mRNA synthesis in Bacillus subtilis. J. Biol. Chem., 247:2289-2296.

Swanton, M. and G. Edlin. 1972, Isolation and characterization of an RNA relaxed mutant of B. subtilis. Biochem. Biophys. Res. Comm., 46:583-589.

Gallant, J., L. Shell and R. Bittner. 1976, A novel nucleotide implicated in the response of E. coli to energy downshift. Cell, 7:75-84.

Constantini, M., R. Zippel and E. Sturani. 1977, Levels of deoxyribonucleotides and rate of RNA synthesis in Neurospora crassa. Exp. Cell. Res., 99:245-252.

Sturani, E., M.G. Constantini, R. Zippel and F. Alberghina. 1976, Regulation of RNA synthesis in Neurospora crassa. Biochim. Biophys. Acta., 476:272-278.

Sturani, E., F. Magnani and F. Alberghina. 1973, Inhibition of rRNA synthesis during a shift-down transition of growth in Neurospora crassa. Biochem. Biophys. Acta., 319:153-164.

Singh, R.P., B. Setlow and P. Setlow. 1977, Levels of small molecules in the forespore of sporulating Bacillus megaterium. J. Bacteriol., 130:1130-1138.

Wiame, J.M. 1947, The metachromatic reaction of hexametaphosphate. J. Amer. Chem. Soc., 69:3146-3147.

Wiame, J.M. 1948, The occurence of physiological behavior of two metaphosphate fractions in yeast. J. Biol. Chem., 178:919-929.

Schmidt, G., L. Hecht and S. Thannhauser. 1946, The enzymatic formation and accumulation of large amounts of metaphosphate in baker's yeast. J. Biol. Chem., 166:775-776.

Harold, F.M. 1966, Inorganic polyphosphates in biology: Structure, metabolism and function. Bacteriol. Rev., 30:772-794.

Sall, T., S. Mudd and J. Davis. 1956, Factors conditioning the accumulation and disappearance of metaphosphate in cells of Corynebacterium diphtheriae. Arch. Biochem. Biophys., 60:130-146.

Winder, F.G. and C. O'Hara. 1962, Effect of iron deficiency and zinc deficiency on composition of Mycobacterium smegmatis J. Biochem., 82:98-108.

Nishi, A. 1960, Enzymatic studies on the phosphate metabolism in germination of spores of Aspergillus niger. J. Biochem., 48:758-767.

Nishi, A. 1961, Role of polyphosphates and phospholipid in germination of spores of Aspergillus niger. J. Bacteriol., 81:10-19.

Goodman, E.M., H.W. Sauer, L. Sauer and H.P. Rusch. 1969, Polyphosphate and other phosphorous compounds during growth and differentiation of Physarum polycephalum. FEBS Let., 15:1325-1331.

Hildebrandt, A. and H.W. Sauer. 1977, Transcription of rRNA in the life cycle of Physarum may be regulated by a specific nucleolar initiation inhibitor. Biochem. Biophys. Res. Comm., 74:466-472.

Ludwig, J.R., S.G. Oliver, and C.S. McLaughlin. 1977, The effects of amino acids on growth and phosphate metabolism in a prototropic yeast system. Biochem. Biophys. Res. Comm., 79:16-23.

Lé John, H.B., L.E. Cameron, R.C. McNaughton and G.R. Klassen. 1977, Diouanosine nucleotides of fungi that regulate RNA polymerases isolated and partially characterized. Biochem. Biophys. Res. Comm., 66:560-567.

McNaughton, R.D., G.R. Klassen, and H.B. Lé John. 1975, Phosphorylated guanosine derivatives of eucaryotes: Regulation of DNA-dependant RNA poylmerase I, II and III in fugal development. Biochem. Biophys. Res. Comm., 66:468-474.

Lé John, H.B., L.E. Cameron, G.R. Klassen and R.U. Meusser. 1978, Effects of L-glutamine and HS compounds on growth and sporulation metabolism of achlya.
Can. J. Biochem., 56:227-236.

Lewis, W.H., R.D. McNaughton, H.B. Lé John and J. Wright. 1976, Regulation of fungal ribonucleotide reductase by unusual dinucleotides.
Biochem. Biophys. Res. Comm., 71:128-135.

Strehler, B.L. and J.K. Totter. 1954, Methods of Biochemical Analysis. vol. I. p. 341-356. Determination of ATP and related compounds: Firefly luminescense.
D. Glick, Ed.
Interscience Publishers, N.Y.

Ames, B.N. 1966, Assay of inorganic phosphate, total phosphate and phosphates.
Methods. Enzymol., 8:115-118.

Cashel, M., R.A. Lazzarini and B. Kalbacher. 1969, An improved method for thin-layer chromatography of nucleotide mixtures containing ^{32}P-labelled ortho phosphate.
J. Chromatogr., 40:103-109.

Brandhorst, B. and D. Fromson. 1976, Lack of accumulation of ppGpp in sea urchin embryos.
Dev. Biol., 48:458-460.

Griffin, J.B., N.W. Davidian and R. Penniall. 1965, Studies of phosphorous metabolism of isolated nuclei: identification of polyphosphate as a product.
J. Biol. Chem., 240:4427-4434.

Okano, R., K. Bacon and E. Rosenberg. 1970, RNA synthesis during microcyst formation in M. xanthus: Characterization of DNA-RNA hybridization.
J. Bacteriol., 104:275-282.

Pao, C.C., J. Paietta and J. Gallant. 1977, Synthesis of ppGpp (MS I) in Saccharomyces cerevisiae.
Biochem. Biophys. Res. Comm., 74:314-322.

Sutherland, I.W. 1976, Novel surface polymer changes in development of Myxococcus spp.
Nature (London), 259:46-47.

Chow, C.T., and I. Takahashi. 1972, Acid-soluable nucleotides in an asporogenous mutant of Bacillus subtilis.
J. Bacteriol., 109:1175-1180.

Terry, K.R. and A.B. Hooper. 1970, Polyphosphate and orthophosphate content of Nitrisomonas europeae as a function of growth. J. Bacteriol., 103:199-206.

Harold, F.M. and S. Sylvan. 1963, Accumulation of inorganic polyphosphates in Aerobacter aerogenes. J. Bacteriol., 86:222-231.