THE ROLE OF β-HYDROXYBUTYRATE DURING ENCYSTMENT OF AXOTOBACTER VINELANDII

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ABSTRACT

THE ROLE OF β-HYDROXYBUTYRATE DURING ENCYSTMENT OF *AZOTOBACTER VINELANDII*

By

Victoria Millicent Koo Hitchins

Vegetative cells of *Azotobacter vinelandii* grow well in the presence of glucose but, upon the replacement of glucose with DL-β-hydroxybutyric acid (BHB) as the sole carbon source, the cells undergo encystment. This process of cellular differentiation results in the development of metabolically dormant cells called cysts. The objective of this thesis was to understand the role and function of BHB as the specific inducer of encystment of *A. vinelandii*.

Encystment occurred over a period of 36 hours and was characterized by an ordered sequence of morphological and biochemical events. It was sensitive to glucose and as little as 0.1% glucose in the encystment medium blocked the morphological development of the cysts (abortive encystment) and altered the time of appearance and the activity of the inducible enzyme BHB dehydrogenase

(E.C.1.1.1.30; D-3-hydroxybutyrate: NAD oxidoreductase). The addition of adenosine 3', 5'-monophosphate (cyclic AMP) to the culture medium did not reverse glucose repression during abortive encystment.

Immediately upon the initiation of encystment, BHB was incorporated and metabolized by the cells. Chemical fractionation of the encysting cells showed that BHB-3-^{14}C was found only in the protein, lipid and ribonucleic acid fractions. Within 30 minutes after the induction of encystment, radioactive-labelled BHB was also found in the phospholipid fraction of the membrane. Cells grown in the presence of glucose, acetate and propanol (vegetative growth) utilized molecular nitrogen, while cells in the presence of BHB or crotonate (encystment conditions) ceased to fix nitrogen within one hour.

Encystment was characterized by a shift from glucose catabolism to gluconeogenesis. This was demonstrated by the decrease in specific activity of glucose-6-phosphate dehydrogenase (E.C.1.1.1.49; D-glucose-6-phosphate: NADP oxidoreductase), a key enzyme of the Entner-Doudoroff pathway of glucose catabolism, and an increase in specific activities of BHB dehydrogenase, isocitrate dehydrogenase (E.C.1.1.1.42; threo-D_s-isocitrate: NADP oxidoreductase (decarboxylating)), isocitrate lyase (E.C.4.1.3.1; threo-D_s-isocitrate glyoxylate lyase) and malate synthase (E.C.4.1.3.2.; L-malate glyoxylate-lyase

(CoA-acetylating)) at 3-6 and 21 hours during encystment. Peak activities of fructose-1, 6-diphosphate aldolase (E.C.4.1.2.13; fructose-1, 6-diphosphate D-glyceraldehyde-3-phosphate-lyase) occurred at 6 and 24 hours and fructose 1, 6-diphosphatase (E.C.3.1.3.11; D-fructose-1, 6-diphosphate 1-phosphohydrolase at 9 and 27 hours.

Macromolecular synthesis was followed during encystment using radioactive-labelled precursors for deoxyribonucleic acid, ribonucleic acid and protein. Deoxyribonucleic acid synthesis ceased just prior to the final cell division at 4-6 hours, but ribonucleic acid synthesis continued until the twelfth hour. From labelling studies and the appearance of new enzyme activities, it appeared that protein synthesis continued throughout encystment to provide the cell with encystment-related enzymes and necessary precursors for the synthesis of cyst coats.

Encysting cells were more resistant than vegetative cells to chloramphenicol. Concentrations of up to 100 µg of chloramphenicol per ml did not alter significantly the rate of L-leucine-U-^{14}C incorporation into encysting cells nor the course of morphogenesis. Disc gel electrophoresis in sodium dodecyl sulfate showed that there was no difference in protein subunits from encysting cells cultivated in the presence or absence of chloramphenicol.

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DEDICATION

To my parents who provided the opportunity and encouragement and to my husband who gave me his patience, understanding, and advice.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Both procaryotic and eucaryotic organisms can undergo cellular differentiation which involves both physiological and morphological changes occurring in an ordered time sequence. The controls which ensure the organization and the regulation of gene activity in the various organisms during these processes of differentiation are not well known.

Bacteria are relatively simple organisms and those species which undergo morphogenesis have been studied as models of this process (27, 49, 91). There are several advantages in using bacteria: (1) they are easy to grow and maintain, (2) they grow on defined media, permitting the correlation of biochemical events with morphological development, and (3) they are easily manipulated to yield mutants which are blocked at various stages of development. The characterization and analysis of such mutants can lead to the determination of the order of morphological and biochemical changes which occur during cellular differentiation. Equivalent biochemical and genetic analysis cannot be readily achieved in the more complex differentiating eucaryotic cells. Thus, studies of the regulatory

mechanisms in differentiating bacteria may provide a valid basis for extrapolation to higher organisms.

Differentiating bacterial species which have been investigated include several species of the spore-forming Bacillaceae family and certain members of the cyst-forming Azotobacteriaceae and Myxococcaceae families. The rod-shaped bacilli can differentiate into spherical dormant bodies called spores which are resistant to heat, dessication and a variety of chemicals. Under appropriate conditions, these spores can germinate to become vegetative rods again. The sequential changes which occur in sporulating cells have been extensively studied and include morphological changes (69), the appearance of new enzymes (38, 49, 64, 70), and the synthesis of spore-specific compounds such as dipicolinic acid (49, 69). Various mutations which do not affect vegetative growth but prevent the development of normal spores demonstrate that these morphological and biochemical changes during sporulation are governed by genetic determinants (49).

Vegetative cells of certain Azotobacter are capable of undergoing encystment to produce metabolically dormant cells called cysts. The cyst is a spherical, thick-walled body and is more resistant than the vegetative cell to physical stresses (dessication, ultraviolet irradiation, sonic treatment). The similarities in the physical properties and morphologies of the spores and cysts suggest there

may be fundamental similarities in the differentiation of the procaryotes. Little is known about the biochemical changes which occur during the encystment and germination of cysts of Azotobacter.

Vegetative cells of Azotobacter species grow rapidly and to high cell concentrations in a medium containing glucose as the carbon source but the cells form few cysts, even when glucose is depleted from the medium (89). On the other hand, cells do not grow well in the presence of n-butanol (111) or its oxidation products crotonate and β-hydroxybutyric acid (BHB) (54) but undergo almost complete encystment. The addition of both glucose and BHB to the medium blocks the development of the cysts (54). This has led to a two-step growth procedure for producing cysts. Vegetative cells are harvested at the end of their exponential growth, washed, and resuspended in fresh buffer and BHB to initiate encystment.

The repression by glucose and the specificity of BHB as an inducer of encystment is superficially similar to the phenomenon of catabolite repression by glucose of β-galactosidase (E.C.3.2.1.23; β-D-galactoside-galactohydrolase) synthesis in Escherichia coli (77). This catabolite repression can be overcome by the addition of adenosine 3', 5'-monophosphate (cyclic AMP) to cells (60, 77, 79). In addition, this nucleotide has been implicated in cellular differentiation by stimulating the

formation of flagella in E. coli and Salmonella typhimurium (116), and inducing stalk formation in Dictystelium discordeum (11). It is not known whether cyclic AMP exerts a control function during encystment in Azotobacter.

The morphogenetic aspects of encystment have been studied using phase-contrast and electron microscopic techniques. Upon induction of encystment with BHB, the motile, rod-shaped vegetative cells undergo cell division (89), become rounded and lose their flagella (40). Lipoprotein spherules or "blebs" from the cell wall surface are excreted into the carbohydrate capsule surrounding the cell. There they coalesce and flatten to form the discontinuous, multilayered sheets of the outer cyst coat (40). This process of excretion of cyst coat components occurs over a 36-hour period after which time the cells are mature cysts. During this same time period, modifications occur which convert the cell into the cyst central body.

BHB is specific for the induction of encystment and promotes a sequence of morphological changes leading to the formation of a dormant cell. However, nothing is known of the mechanism(s) by which this compound exerts its effects. A unique sequence of biochemical events must occur during this cellular differentiation process. Preliminary studies on the physiology of encystment have shown that very soon after the initiation of encystment radioactive-labelled BHB is incorporated into the cells and $^{14}CO_2$ is released

(54). In addition, cells cease to utilize molecular nitrogen and BHB dehydrogenase is induced (89) to catalyze the oxidation of BHB to acetoacetate (43).

The objective of the research presented in this thesis was to understand the role and function of BHB as a specific inducer of encystment in A. vinelandii by attempting to correlate the morphological changes with biochemical events. Previous studies of the morphological changes (40) and the chemistry of the cyst coats (55, 56) suggested that conversions of lipid (BHB) to carbohydrates and proteins were important during encystment. Therefore, the time course for the appearance of enzymes of BHB metabolism and gluconeogenesis has been determined to ascertain whether encysting cells had such biochemical capabilities.

LITERATURE REVIEW

Azotobacter vinelandii is a member of the Azotobacteriaceae family which has only one genus, *Azotobacter*. The six species of *Azotobacter* include *A. beijerinckii*, *A. chroococcum*, *A. vinelandii*, *A. agilis* (*agile*), *A. insignis* (*insigne*), and *A. macrocytogenes*. They are all Gram-negative, obligate aerobes, found in soil and water, and utilize molecular nitrogen. Certain members of this genus are able to undergo a cyclic process of cellular differentiation. The rod- or peanut-shaped vegetative cells can be induced to form rounded specialized resting cells called cysts, which can, in the presence of a suitable substrate, germinate and become vegetative cells again.

A. vinelandii was first isolated and described by Lipman in 1904 (58) and is characterized by a relatively large rod or peanut-shaped vegetative cell with an average individual cell size of 2.4 μm x 0.75 μm. This organism is peritrichously flagellated (4), produces a water-soluble pigment which fluoresces apple-green under ultraviolet light (41, 71) and can undergo encystment.

Metabolism and Cytology of Vegetative Cells

Carbohydrate Metabolism

Vegetative cells of A. vinelandii may be cultivated in sucrose or glucose as the carbon source. Other substrates which are readily utilized are fructose, galactose, and maltose. Enzyme studies (66, 67) and radiorespirometric data (99) indicate that the Entner-Doudoroff pathway is the major route for glucose catabolism while the pentose phosphate shunt is a minor pathway (99). All the enzymes of the Embden-Meyerhof-Parnas pathway except phosphohexokinase (E.C.2.7.1.11; ATP: D-fructose-6-phosphate 1-phosphotransferase) have been detected in A. vinelandii suggesting that glucose is not catabolized by this pathway (66).

Tricarboxylic Acid Cycle

A. vinelandii cells also grow well on acetate and substrates of the tricarboxylic acid (TCA) cycle (32, 57, 101, 108) indicating that the organism has an active TCA cycle. The enzymes succinic dehydrogenase (E.C.1.3.99.1; succinate: (acceptor) oxidoreductase) and malic oxidase (E.C.1.1.3.3; L-malate: oxygen oxidoreductase) are found to be associated with the cell membrane while aconitase (E.C.4.2.1.3; citrate (isocitrate) hydro-lyase), nicotinamide adenine dinucleotide phosphate (NADP)-linked

isocitrate dehydrogenase (E.C.1.1.1.42; threo-D_s-isocitrate: NADP exidoreductase (decarboxylating)), a-ketoglutaric oxidase (E.C.1.2.4.2; 2-oxoglutarate: lipoate oxidoreductase (acceptor-acylating)) and fumarase (E.C.4.2.1.2; L-malate hydro-lyase) are soluble enzymes (2). It has been suggested by several investigators (5, 6) that one of the functions of isocitrate dehydrogenase may be to provide the necessary reduced nucleotides utilized during nitrogen fixation. A. vinelandii also possesses a pyridine nucleotide transhydrogenase (E.C.1.6.1.1; reduced-NADP NAD oxidoreductase) (65) which can catalyze the reversible transfer of hydrogen between the pyridine nucleotide coenzymes: $NADPH + NAD^+ + NADP^+ + NADH$. Since there is a complete coupling between oxidation and phosphorylation in intact Azotobacter cells, the oxidation of one molecule of NADH can lead to the formation of 3 molecules of adenosine triphosphate (ATP), independent of the presence of a nitrogen-fixing system (20).

β-hydroxybutyrate Metabolism

A variety of bacterial genera which include Azotobacter, Bacillus, Rhizobium, Actinobacillus, Spirillum, Vibrio, Chronobacterium, and Micrococcus (25, 33, 100, 108) can synthesize the storage polymer poly-β-hydroxybutyric acid (PHB), a polymeric ester of

D-(-)-β-hydroxybutyric acid, when cells are grown on glucose, acetate or succinate (94). The polymer is synthesized in the following manner. Pairs of acetate molecules, in the form of acetyl coenzyme A, are combined to form acetoacetate. This compound is then reduced by the enzyme acetoacetyl CoA reductase (E.C.1.1.1.36; D-3-hydroxyacyl-CoA: NADP oxidoreductase) to form β-hydroxybutyryl CoA, the substrate for PHB granule-bound poly-β-hydroxybutyrate synthetase (47, 84). The necessary ATP and reduced nucleotides utilized during the formation of PHB are supplied by the oxidation of glucose or acetate via the Entner-Doudoroff and TCA pathways (90). Substrates which promote the synthesis of PHB appear to promote encystment in Azotobacter cells (97).

Nitrogen-Fixation

Previous interest in the Azotobacteriaceae was based on the various organisms' ability to utilize molecular nitrogen when provided with a suitable energy source. Although many other microorganisms can fix nitrogen (the blue-green algae, purple and green bacteria and certain members of the genera Clostridium and Desulfovibrio), only the Azotobacter cells can incorporate greater than 15 μg nitrogen per gram of glucose, sucrose, or mannitol (41). However, Azotobacter cells do not fix nitrogen when they are grown in the presence of metabolizable nitrogen-containing compounds such as ammonia, nitrate, urea, and asparagine (110).

A more thorough review on the biochemistry of nitrogen fixation is presented elsewhere (23) and only a brief summary will be given here. The membrane-bound nitrogen-reducing enzyme complex, nitrogenase, is composed of two separable metallo-protein components (Fractions 1 and 2) and both are essential for nitrogen-fixation. Fraction 1 (molybdoferredoxin) is a protein of molecular weight of about 270,000 daltons and contains 2 atoms of molybdenum, 32 to 34 atoms of iron atoms and 26 to 38 labile sulfide groups per molecule (14). Fraction 2 (azoferredoxin) has a molecular weight approximately 40,000-55,000 daltons and contains 4 iron and 4 labile sulfide groups (13, 14, 81). The purified components of nitrogenase from Azotobacter is sensitive to oxygen (22, 26, 75), requires magnesium, ATP (36) and reduced nucleotides (6, 115), and may be coupled to the enzyme NADH dehydrogenase (E.C.1.6.99.3: reduced-NAD: (acceptor) oxidoreductase) (114).

Nitrogenase can also reduce other triply bonded substrates such as acetylene to ethylene, and cyanide to methane, ammonia and methyl amine (13). Since ethylene can be easily detected by gas chromatography (98), the rate of acetylene reduction by Azotobacter cells in an nitrogen-free atmosphere is used as an index of its nitrogen fixing capacity. The rate of nitrogen-fixing activity should be one-third of the acetylene-reducing

activity since the reduction of N_2 to ammonia requires the transfer of 6 electrons whereas the reduction of acetylene to ethylene requires 2 electrons. The ratio, however, is in the range of 1: 3.5 since there is more concomitant ATP-dependent H_2 evolution during N_2-fixation than during acetylene reduction (23).

Morphology

The morphology and cytology of the vegetative cells of Azotobacter were studied by Pochon et al. (80), Tchan et al. (102) and Winogradsky (111) who observed that only the vegetative cells are rod-shaped and motile while the older cells are oval and non-motile. Electron micrographs (40, 50) show that the vegetative cells contain deoxyribonucleic acid (DNA) strands and ribosomal particles in the finely granular cytoplasm. Within the cytoplasm and along the cell periphery is a network of membranous vesicles believed to be associated with the nitrogenase system as they are present when cells are grown with atmospheric nitrogen (74). Vegetative cells in stationary phase are surrounded by a dense fibrous capsule which is synthesized at the surface of the cell wall (18). Cohen and Johnstone (21) analyzed the extra-cellular material and found it to be composed of galacturonic acid, α-D-glucose and rhamnose in the ratio of approximately 43: 2: 1, and a hexuronic acid lactone believed to be mannuronolactone. Proctor (82)

suggested that the capsular material could serve as both a carbon and energy source for the cells.

Differentiation in the Procaryotes

Certain groups of bacteria are able to give rise to dormant or resting cells following exponential growth and the apparent depletion of growth substrates. These resting bodies include endospores (spores) which are produced by the Gram-positive rods of the genera Bacillus and Clostridium, and by the coccus Sporosarcina, and cysts which are produced by the genera Azotobacter and Myxococcus. Both cysts and spores are rounded, phase-bright resting cells which are reasonably similar when viewed in the light microscope (9). Their similarity extends to their being able to withstand certain adverse environmental conditions and hence there may be similarities in the controls occurring during differentiation of these procaryotes.

Sporulation

The sporulation process in the Bacillaceae begins in the cells at the end of exponential growth when glucose is depleted from the medium and the cells begin to metabolize acetate or other organic acids which have resulted from glucose metabolism. The rod-shaped vegetative cells undergo a well-defined sequence of morphological, biochemical and physiological changes to

become spores whose morphology and physical properties differ from vegetative cells. The spore is a thick-walled resting cell which is refractile under phase-contrast microscopy, and is more resistant to heat, dessication, sonic treatment, ultraviolet irradiation and certain deleterious chemicals than its vegetative cell.

The morphological development of the endospore can be observed by electron microscopy and has been divided into seven stages: (1) replication and alignment of the DNA (axial filament), (2) formation of the sporulation septum to separate the mother cell from the germ cell (septum formation), (3) engulfment of the germ cell by the mother cell (engulfment of the forespore), (4) cortex formation, (5) coat formation, (6) maturation of the spore (completion of the coats), and (7) lysis of the mother cell or sporangium to release the spore (69). The net result of sporulation is a small germ cell surrounded by a cortical layer and a multilayered coat. The spore coat is believed to be unique to the spore as it is synthesized after stage 1 (49). By stage 6, the spore is refractile and resistant to lysozyme (E.C.3.2.1.17; mucopeptide N-acetyl-muramylhydrolase) and heat (49).

Endospore formation is also characterized by changing enzyme patterns and active turnover of RNA and protein. During sporulation enzymes of the TCA (37, 38, 70) and glyoxylic acid cycles (64), and the enzymes

involved in the metabolism of PHB (47) are induced to provide the cell with energy and precursors for spore components. Until recently, it was believed that the appearance of the TCA cycle enzymes was required for sporulation since B. subtilis mutants lacking aconitase did not sporulate (37, 38). Recently, however, Carls and Hanson (17) isolated mutants of B. subtilis which were lacking a functional TCA cycle but were able to sporulate. Thus, the actual function(s) of the TCA cycle in sporulation remains unknown. Turnover of the proteins provides the developing spore with the amino acids for the synthesis of the enzymes necessary for sporulation, and is also hypothesized to permit the cell a means to eliminate superfluous vegetative cell enzymes (49).

Encystment

Although the inducer for the initiation of sporulation is not known, n-butanol (68, 111), crotonate or BHB (54) will specifically induce encystment in A. vinelandii and A. chroococcum. Encystment is sensitive to the presence of glucose which represses the initiation of the differentiation process or, when added to the encystment medium, blocks morphological development (abortive encystment). Abortive encystment is characterized by the release of a viscous polymer into the medium (54). This polymer is a water-soluble, noncross-linked peptidoglycan material which can be cleaved with lysozyme

and lysostaphin. Furthermore, electron micrographs reveal that during this block in development, the cyst outer coat, the exine, is not associated with the central body.

Cyst Properties

Like the spores, cysts have morphological and physical properties which are different from vegetative cells. They are also thick-walled, spherical bodies which are metabolically dormant, refractile under phase-contrast microscopy, and are more resistant to dessication, sonic treatment and ultraviolet irradiation (95, 96). However, as might be expected, cysts differ from spores in some aspects. Cysts are not more heat resistant than vegetative cells nor do they contain 2, 6-pyridine dicarboxylic acid (dipicolinic acid) (113), a chelating agent. Dipicolinic acid (95) or ethylenediaminetetraacetic acid (EDTA) in the absence of lysozyme can lyse cysts but not vegetative cells (34, 35). Lastly, the loss of calcium from the cysts does not decrease their viability nor their resistance to dessication (93).

Cyst Morphology

The fine structure of the cyst has been studied using a variety of preparative techniques for electron microscopy. Cagle et al. (16), Hitchins and Sadoff (40), Lin and Sadoff (54), Parker and Socolofsky (76), Socolofsky and Wyss (95), Tchan et al. (102), Vela et al.

(105) and Wyss et al. (113) have all suggested that the cyst, measuring 1.5 μm to 2.0 μm in diameter, is a modified vegetative cell surrounded by two layers, the intine and the exine. Using the technique of ultrathin sections for electron microscopy, the DNA strands, ribosomal particles and PHB granules can be observed in the cytoplasm of the central body of the cyst (40, 54, 76, 95, 102, 113). Surrounding the central body is the electron-transparent material of the intine, which in turn is surrounded by an electron-dense exine (40, 54, 95, 102, 113). Ultrathin sections (40, 54, 95, 105, 113), metal shadowing (56), freeze-cleaving (48), and freeze-etch (16) techniques have all demonstrated that the exine coat is composed of several overlapping layers, each measuring 6 to 7 nm thick.

Morphogenesis

The morphological changes which occur during encystment have also been studied by electron microscopy. Encystment occurs over a much longer time period than sporulation and thus it is not as easily divided into defined stages as is sporulation. Before encystment can proceed, however, the synthesis and presence of the capsular material surrounding vegetative cells at the end of exponential growth seems to be necessary for the formation of mature cysts. A non-capsulated mutant of A. vinelandii has been reported to be unable to form cysts (28).

Wyss et al. (112), using $KMnO_4$ as a fixative, noted that vegetative cells of A. vinelandii became spherical and were surrounded by a fibrous capsule. Fragments of exine were then laid along the outer edges of this capsular material while the intine appeared as the area between the central body and exine.

Hitchins and Sadoff (40) studied the progressive transition from vegetative cells to cysts by electron microscopy of negative-stained and OsO_4-fixed cells since fine structural details cannot be seen when $KMnO_4$ is used for fixation. The vegetative cells were rod-shaped and motile when viewed under phase-contrast microscopy and were peritrichously flagellated when negative-strained with phosphotungstic acid. The first detectable morphological change was the conversion of the rod-shaped vegetative cells after cell division to rounded cells or "precysts" (40). The use of OsO_4 as a fixative to preserve biological structures, especially membranes, allowed Hitchins and Sadoff (40) to describe the morphogenesis of the cyst outer coat. As encystment proceded, membranous components were excreted from the cell wall surface as vesicles or "blebs" into the surrounding capsular material. Twelve hours after the cells had been induced to encyst, these blebs appeared to have coalesced and flattened to form individual fragments within the inner edge of the capsular material. These membrane-like fragments measured 115 to

355 nm long and 6 to 7 nm wide, which are the cross-sectional dimensions of the exine layers of the mature cyst. The number of blebs and flattened fragments produced per cell increased up to 30 hours after which time they decreased as the cyst became mature and the exine layers were more compact and dense along the outer edge of the capsular material. At this time, the central body appeared more compact and the number of PHB granules per cell was reduced. The appearance of blebs during encystment was also observed by Cagle et al. (16) who used the technique of freeze-etching for electron microscopy.

Composition of the Cyst Coats

The two cyst coats, the exine and the intine, have been fractionated and isolated for chemical analyses (55, 56). Cysts can be disrupted in 10^{-3} M solution of EDTA, pH 7 (35), and the central body, exine and intine layers can be isolated according to their buoyant density differences in sucrose gradients. The purified exine and intine materials differ significantly with respect to their chemical composition. The intine consists of 44% carbohydrate, 9.1% protein, and 37% lipid. The exine is comprised of 32% carbohydrate, 28% protein, and 30% lipid. The carbohydrates of both the exine and intine contain glucose, mannose, xylose and rhamnose while the exine has in addition glucosamine and galactosamine. Hydrolysates

of the exine contain a wide range of amino acids including large amounts of glutamic acid, glycine, alanine and lysine, which are also found in other Gram-negative bacterial cell walls. The lipid in the exine is primarily bound (extractable after acid hydrolysis) while in the intine it is mostly free (extractable by ether). The principal phospholipids detectable by thin layer chromatography are phosphatidyl ethanolamine and diphosphatidyl glycerol.

Cyclic AMP

The role and function of BHB as the specific inducer of encystment in A. vinelandii is not well understood. The specificity of BHB as an inducer and the repression of encystment by glucose is similar to the phenomenon of catabolite repression. Thus in attempting to assign a role for BHB in encystment, it is necessary to consider the possibility that it may act in concert with cyclic AMP. Cyclic AMP can overcome glucose repression of the synthesis of a variety of inducible enzymes such as β-galactosidase, galactoside permease, arabinose permease, galactokinase (E.C.2.7.1.6; ATP; D-galactose 1-phosphotransferase), glycerolkinase (E.C.2.7.1.30; ATP; glycerol phosphotransferase), serine deaminase (E.C.4.2.1.13; L-serine hydro-lyase (deaminase)), and tryptophanase synthesis (E.C.4.2.1.20; L-serine hydro-lyase (adding indole)) (77). Glucose and certain other metabolites

apparently inhibit synthesis of these inducible enzymes by reducing the level of cyclic AMP within the cell (60).

Cyclic AMP is involved in other functions besides the stimulation of inducible enzyme syntheses. The nucleotide is required for the formation of flagella (and, hence, motility) in cyclic AMP-deficient mutants of E. coli and S. typhimurium (116). Cyclic AMP seems to play two main roles in the differentiation of D. discoideum, a primitive eucaryotic organism. High concentrations of the nucleotide initiate aggregation of the slime mold amoebae into a mass of cells which coalesce to form a multicellular pseudoplasmodium. Later, in the life cycle of D. discoideum, cyclic AMP induces certain cells to differentiate into stalk cells (11).

The mechanisms of action of cyclic AMP on gene activity, in particular the lactose (lac) operon, has recently been investigated. Cyclic AMP acts at the transcriptional level to stimulate the synthesis of β-galactosidase-specific messenger RNA (m-RNA) (19, 29, 77, 79). The nucleotide binds to a specific cyclic AMP-receptor protein which together bind and alter the structure of the lac promoter region. This allows the RNA polymerase to bind to DNA and stimulates transcription of the lac operon (19). Cyclic AMP-receptor protein alone, however, cannot compete with the repressor, and both

cyclic AMP and its receptor protein are required for optimal synthesis of the enzyme (19, 29, 77, 79).

Biochemical Studies of Encystment

Preliminary studies concerning encystment in A. vinelandii (89) suggest that this cellular differentiation process is characterized by an extensive modification of the biochemical activities of its cells. Nitrogen-fixation, measured by the microkjeldahl method, ceases and the enzyme BHB dehydrogenase is induced within one hour after BHB is added to the medium. DNA synthesis diminishes and stops after a final round of cell division. The final DNA content per cell remains constant throughout encystment at 3.4×10^{-14} g per cell.

MATERIALS AND METHODS

Strain and Cultivation

Azotobacter vinelandii ATCC 12837, which was used throughout these experiments, was cultivated in Burk's nitrogen-free buffer (75) and 1% glucose at 30 C on a rotary shaker. At the end of exponential growth, 18-22 hours, cells were harvested, washed and resuspended in fresh Burk's buffer and 0.2% DL-BHB to initiate encystment. After 36 hours, more than 90% of the cells became mature cysts. Addition of both 0.1% or 0.2% glucose and 0.2% BHB to the buffer promoted abortive encystment. Burk's buffer and the carbon sources were autoclaved or filter-sterilized separately.

Chemicals

Acetylene (purified grade), ethylene and a gas mixture of O_2 (22%), CO_2 (0.04%) and argon (78%, high purity) were all obtained from Matheson. Sodium DL-3-hydroxybutyrate-3-^{14}C was purchased from Amersham/Searle (Arlington Heights, Ill.). L-leucine-U-^{14}C was a product of CalAtomic (Los Angeles, Calif.) and potassium DL-3-hydroxybutyrate-3-^{14}C and uracil-2-^{14}C were from New England Nuclear (Boston, Mass.). Coenzyme A, nicotinamide-adenine dinucleotide (NAD), nicotinamide-adenine

dinucleotide phosphate (NADP) and adenosine 3', 5'-monophosphate (cyclic AMP) were all purchased from CalBiochem (San Diego, Calif.). The sodium salt of DL-β-hydroxybutyrate, sodium dodecyl sulfate (SDS) and chloramphenicol were obtained from Sigma (St. Louis, Mo.). Acrylamide; N,N'-methylenebis acrylamide (methylenebis acrylamide); N, N, N'-tetramethylethylene diamine (TEMED) were purchased from Eastman Organic Chemicals (Rochester, N.Y.). Coomassie brilliant blue was from Mann Research Laboratories, New York, N.Y.) and the sodium salt of bromphenol blue was a product of Allied Chemical (New York, N.Y.). All other materials used were of the highest grade obtainable.

Incorporation of Radioactive-Labelled BHB into Cysts

BHB-3-^{14}C (0.5 μCi; 11.8 mCi/mmole) was added to cells in 100 ml of Burk's nitrogen-free buffer and 0.2% unlabelled BHB to initiate encystment. Radioactive CO_2 was trapped on Whatman #1 filter paper saturated with 10% KOH. Cells were collected on 0.45-μm membrane filters (Gelman, Ann Arbor, Mich.) and washed 3 times with fresh Burk's buffer containing 0.2% BHB. The cell-free medium and the washes were combined and counted for radioactivity. Bray's scintillation fluid (12) was added to the cell-free medium while a toluene-based scintillation fluid (15) was used for the non-aqueous samples. Radioactivity of the

three fractions was measured with a Packard Model 3320 Tri-carb liquid scintillation spectrometer, and the results were corrected for quenching and plotted on a per-ml-sample basis.

Distribution of Radioactive-Labelled BHB and L-leucine into Cyst Fractions

BHB-3-^{14}C (10 μCi; 4.52 mCi/mmole) was added to cells in 100 ml of Burk's nitrogen-free buffer and 0.2% unlabelled BHB. Alternatively, L-leucine-U-^{14}C (5 μCi; 216 mCi/mmole) was added to washed vegetative cells in the presence of 0.2% BHB and 5 μmole unlabelled L-leucine per ml. The distribution of the radioactivity into the protein, DNA, ribonucleic acid (RNA), lipid and acid soluble fractions was determined by the membrane fractionation method of Roodyn and Mandel (86). This method involved extraction of the cells with ethanol, with cold trichloracetic acid (TCA), with hot TCA and with NaOH, followed by filtration through membrane filters. The distribution of the radioactivity in the DNA, RNA, lipid, protein and acid-soluble fractions was then calculated by difference.

Assay for Poly-β-Hydroxybutyrate Granules

The method of Stockdale et al. (100) was used to assay poly-β-hydroxybutyrate (PHB) which accumulates in encysting cells. The assay of PHB involved digesting the

cells with alkaline hypochlorite, followed by washing the resulting residue containing PHB with distilled water, acetone, and diethyl ether. The polymer was then dissolved in concentrated sulfuric acid and read at 235 nm. The amount of PHB was expressed as the µg PHB per µg cell nitrogen. The nitrogen content of the encysting cells was determined by the microkjeldahl method (103).

Assay for Nitrogen Fixation

The acetylene reduction technique of Stewart et al. (98) was used as an index of the rate of nitrogen fixation. At regular time intervals, triplicate 2-ml samples of vegetative or encysting cultures were added to 5-ml capacity glass serum bottles. The samples were flushed with an O_2-CO_2-Ar gas mixture prior to fitting the bottles with rubber serum bottle stoppers. One-half milliliter of acetylene was then injected into each bottle and the samples were incubated for 1 hour at 26 C. One bottle immediately received 0.3 ml of 2% $HgCl_2$ and served to establish the background of ethylene in acetylene. Acetylene reduction was terminated by the addition of $HgCl_2$ to the cells. Ethylene formation was detected by gas chromatography with a Varian-Aerograph model 600D gas chromatograph equipped with a flame detector and a 1 m column containing Porapak N, and run at 45 C. The carrier gas was N_2 at a flow rate of 25 ml/min. Duplicate 0.5-ml

gas samples were taken from the head space of each serum bottle and injected into the column. The relative rate of nitrogen fixation was expressed as the nmoles of ethylene produced per μg cell nitrogen per hour. The nitrogen content of vegetative and encysting cells was determined by the microkjeldahl method (103).

Addition of Cyclic AMP to Vegetative, Encysting, and Abortive Encysting Cells

Cells of A. vinelandii were cultivated on 2% agar plates containing Burk's nitrogen-free buffer and 1% or 0.1% glucose for vegetative growth; 0.1% glucose and 0.2% BHB, or 0.2% glucose and 0.2% BHB for abortive encystment; and 0.2% BHB for encystment. One set of plates received cells which were pretreated with 0.12 M tris(hydroxymethyl) aminomethane (Tris)-buffer (pH 8) containing 0.5% glycerol and 10^{-3} M sodium phosphate, and 10^{-3} M EDTA for 2 minutes at 30 C to increase the cells' permeability to cyclic AMP (52, 78). A small disc saturated with 10^{-2} M cyclic AMP was placed in the center of each plate which was then put in an incubator at 30 C. Similarly, liquid cultures of vegetative, encysting and abortive encysting cells which were either pretreated or untreated with Tris-EDTA were also cultivated in the presence or absence of 10^{-3} M cyclic AMP to see if abortive encystment could be overcome by the exogenous addition of cyclic AMP.

Preparation of Cell-Free Extracts

Packed cells or cysts were resuspended in 10^{-2} M Tris-HCl buffer (pH 7.5) and were disrupted for 2.5 minutes in a sonic oscillator (Measuring and Scientific Equipment, Ltd.) and then clarified by two cycles of sedimentation at 49,000 x g for 20 and 40 minutes, respectively. The resulting supernatant solution was assayed directly for enzyme activity. It was assumed that all the enzymes studied had approximately the same stability in the cell-free extracts and that the specific activity of any one enzyme at a particular time was a reasonable representation of its intracellular concentration.

Radioactive cell-free extracts were prepared from encysting cells in the presence and absence of 100 µg chloramphenicol per ml. These were used to study the effect of the antibiotic on the incorporation of L-leucine-U-^{14}C into protein subunits. The extracts were prepared by resuspending packed cells in 10^{-2} M sodium phosphate buffer (pH 7) and sonicating for 2.5 minutes. Unlyzed cells and cellular debris were removed by sedimentation at 45,000 x g for 45 minutes.

Protein Determination

The determination of protein in the cell-free extracts was by the procedure of Lowry et al. (59) using bovine serum albumin V as a standard.

Enzyme Assays

The results of the enzyme assays are the composite of four separate experiments. All specific enzyme activities are expressed as units of enzyme per mg protein. Spectrophotometric assays were conducted using a Perkin-Elmer double beam spectrophotometer equipped with a Sargent Recorder, Model SR.

Glucose-6-phosphate dehydrogenase (E.C.1.1.1.49; D-glucose-6-phosphate: NADP oxidoreductase) activity was assayed by following the reduction of NADP (24) at 26 C. BHB dehydrogenase (89) and isocitrate dehydrogenase (46) were measured by recording the reduction of NAD in the presence of the appropriate substrate for each enzyme at 37 C. One unit of enzyme was defined as that amount which reduced 1 μmole of NAD or NADP per minute using a molar absorbance coefficient for NADH or NADPH of 6.22×10^6 cm^2/mole at 340 nm.

Isocitrate lyase (E.C.4.1.3.2; L-malate glyoxylate-lyase (CoA-acetylating)) was measured spectrophotometrically by monitoring the deacylation of acetyl coenzyme A (30). Acetyl CoA was synthesized from CoA by the procedure described by Ochoa (72). The molar absorbance coefficient of acetyl CoA at 232 nm is 4.5×10^6 cm^2/mole.

Fructose-1, 6-diphosphate aldolase (E.C.4.1.3.13; fructose-1, 6-diphosphate D-glyceraldehyde-3-phosphate-lyase) was assayed by measuring colorimetrically the

formation of 2, 4-dinitrophenyl-hydrazine derivative of the triose phosphates formed in 15 minutes at 38 C (92). One unit of enzyme produced an absorbancy of 1.0 at 546 nm under the conditions of the experiment.

Fructose-1, 6-diphosphatase (E.C.3.1.3.11; D-fructose-1, 6-diphosphate 1-phosphohydrolase) (FDPase) was assayed according to the procedure of Rosen et al. (87). The amount of inorganic phosphate (P_i) liberated was proportional to the amount of enzyme added and was determined by the Fiske-Subbarow method (31). One unit of FDPase is that amount of enzyme catalyzing the release of 1 µmole of P_i per minute at 26 C.

Protein Synthesis

Protein synthesis was estimated in cells cultivated in the presence of 5 µmole L-leucine per ml and a carbon source for vegetative growth or encystment by measuring the incorporation of L-leucine-U-^{14}C (5-10 µCi; 216 mCi/mmole) into TCA-insoluble precipitates (86). These were collected on 0.45-µm membrane filters. The filters were washed three times with 5% cold TCA with 5 µmole L-leucine per ml, dried, and then placed into counting vials containing toluene-based scintillation fluid (15).

One set of cells in addition received varying concentrations of chloramphenicol. The extent of inhibition by chloramphenicol on the growth rate was monitored by measuring turbidity (absorbancy) at 600 nm,

and on the morphological development by examining the cells with a Zeiss phase-contrast photomicroscope.

Deoxyribonucleic Acid Synthesis

Deoxyribonucleic acid (DNA) was monitored in cells grown in the presence of uracil-2-^{14}C (21.5 μCi; 45 mCi/mmole), 45 μmole uracil and a carbon source for vegetative or encysting cells. One ml samples were incubated with 0.1 ml 5.5 N NaOH overnight at 37 C to hydrolyze RNA (86). The samples were then cooled in an ice bath and neutralized with 0.1 ml 6 N HCl. Cold 10% TCA was added to make the final concentration 5%. After 30 minutes, the samples were filtered and washed three times with 5% cold TCA containing 5 μmole uracil per ml, dried and counted.

Ribonucleic Acid Synthesis

Ribonucleic acid (RNA) was measured by the incorporation of uracil-2-^{14}C (10 μCi; 22 mCi/mmole) (86) in the presence of 20 μg cytosine per ml to minimize the incorporation of radioactivity into the DNA (51), and the appropriate substrate for vegetative growth or encystment. TCA-insoluble precipitates were collected on membrane filters which were then washed, dried and counted.

Protein Subunit Analysis by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Subunit analysis of the proteins made during encystment in the presence or absence of chloramphenicol was performed by a modification of the method reported by Weber and Osborn (107).

Sodium dodecyl sulfate (SDS)-protein complex was prepared by heating 1 ml of the cell-free extract with 1 ml 10% SDS (v/v) and 0.2 ml β-mercaptoethanol at 100 C for 10 minutes.

SDS-polyacrylamide gels (10%) were prepared from two stock solutions which were stored in brown bottles at 4 C.

Solution A	Solution B
20% acrylamide	0.28% ammonium persulfate
0.735% methylenebis acrylamide	

Freshly made gels consisting of 8 ml of solution A, 4 ml of solution B, 2 ml of 0.8 M sodium phosphate buffer (pH 7), 0.16 ml of 10% SDS, 0.0046 ml of TEMED and 2 ml of deionized distilled water were carefully mixed and pipetted into eight clean glass tubes (10 cm long with an inside diameter of 0.6 cm). Each gel was then covered with a few drops of distilled water. After 30 minutes at room temperature, an interface was observed indicating the gels had solidified. The water from the top of each gel was

gently sucked off and the tubes were then placed in the electrophoresis apparatus. Both the upper and lower chambers of the electorphoresis apparatus each held 500 ml of 0.1 M sodium phosphate buffer (pH 7) containing 0.1% SDS. The gels were then prerun for one hour with a current of 4 ma per gel tube.

For each gel, 100 µg of protein from the SDS-protein mixture, 3 µl of 0.05% bromphenol blue (in water) and two drops of glycerol were carefully mixed and then layered on the top of each gel. Electrophoresis was performed at a constant current of 8 ma per gel with the positive electrode in the lower chamber. After 4.5 hours, the tracking dye, bromphenol blue, reached the end of the gel and electrophoresis was stopped. The gels were removed from the glass tubes by submerging them first in ice water for a few minutes and then gently squirting water from a syringe between the gel and the glass tube wall. One set of gels from each sample was then placed in test tubes and stored at -10 C, while the other set was prepared for staining.

The gels to be stained were first fixed overnight in 10% TCA and 33% methanol before they were stained with 0.4% Coomassie brilliant blue in 10% TCA and 33% methanol. After 10 hours at room temperature in the dark, the gels were rinsed several times with a 10% TCA and 33% methanol

destaining solution overnight at room temperature in the dark. When areas of the gel without protein were colorless the gels were then stored in 10% TCA in the dark at 4 C.

The stained gels were scanned at 550 nm at the speed of 1 cm per minute with a Gilford 240 spectrophotometer equipped with a linear gel transport and a Sargent Recorder, Model SR.

The frozen gels were sliced into 1 mm thick slices with a multiple razor blade gel slicer (Diversified Scientific Instruments, San Leandro, Calif.). Each gel slice was placed in a counting vial and the gel was oxidized with 0.2 ml of 30% H_2O_2 overnight at 70 C. The vials were then cooled to room temperature and 0.2 ml of deionized distilled water was added to each vial, followed by the addition of 5 ml of Bray's scintillation fluid (12). The vials were counted for radioactivity in the Packard Tri-Carb liquid scintillation spectrometer.

Phospholipid Analysis

An analysis of the phospholipids in encysting cells was made by following the one-step extraction and separation method of Bertsch et al. (8) to determine the extent of BHB incorporation into this cell fraction.

Cells of A. vinelandii were induced to encyst in 100 ml of Burk's nitrogen-free buffer containing 0.2% BHB and BHB-3-^{14}C (10 μCi; 4.52 mCi/mmole). At regular time

intervals, one ml samples were removed and were assayed for total radioactivity incorporated into the cells. The remainder of the cells were harvested by centrifugation at 20,000 x g for 15 minutes. The packed cells were then weighed and stored as a paste at -10 C.

The phospholipids were extracted and separated from 0.2 g of packed cells in a one-phase solvent containing sodium acetate buffer (pH 4), chloroform and methanol (1:1:2.2). The phospholipid extract was dried with an Evapo-Mix (Buchler Instruments) and then resuspended in 50 µl of a chloroform-methanol-acetic acid-water solvent (100:50:14:6, v/v). Ten µl was dried on a membrane filter in a vial containing a toluene-based scintillation fluid (15) and counted for radioactivity. Twenty µl of phospholipids in the solvent were then separated and analyzed by thin-layer chromatography (TLC) with Silica Gel G plates (250 microns thick, Analtech, Inc., Newark, Del.) in the following solvents (v/v): (1) chloroform-methanol-acetic acid-water (100:50:14:6), and (2) diisobutylketone (2, 6-dimethyl-4-heptanone)-acetic acid-water (12:5:1) (7).

The phospholipids on the plate were located by ninhydrin (0.25% ninhydrin in acetone-lutidine 9:1, v/v) (62) to detect phospholipids with amino groups, and by the mercury-molybdate reagent of Vasovsky and Kostetsky (104) for phospholipids. Autoradiograms of the radioactive

chromatograms were made with Kodak X-ray film (blue-sensitive), which were developed and fixed with Kodak liquid X-ray developer and Kodak X-ray fixer.

An approximate ratio of radioactivity in the total phospholipid fraction to the total radioactivity incorporated into the cells was determined in the following manner. The amount of radioactivity in cells from 1 ml of culture growing in Burk's medium was determined and multiplied by the number of ml of culture required to yield 0.2 g of packed cells. The ratio of radioactivity in the phospholipid fraction to the radioactivity into cells was based on the average of two separate experiments and two extractions per sample.

RESULTS

β-Hydroxybutyric acid could function in a variety of ways to promote encystment of A. vinelandii. It could be used as an energy source and as a unique substrate for the synthesis of cyst components whose syntheses would require the induction of encystment specific enzymes. Alternatively, BHB could be incorporated directly into cell structures such as membranes to produce modifications of their function whose consequences are the biochemical events of encystment. A series of experiments designed to test these two hypotheses were carried out and their results suggest that both are substantially correct.

Distribution of BHB-e-^{14}C Into Cysts

Radioactive BHB was used to determine the extent of the incorporation and metabolism of the fatty acid in the cell, and to observe the distribution of BHB into various chemical fractions of the encysting cell. Immediately upon the addition of BHB to washed vegetative cells at the end of their exponential growth, the compound was incorporated and metabolized by the cells. The latter was evident by the release of radioactive CO_2 from the culture suspension. At the completion of encystment, 36-40 hours,

approximately 10% of the added BHB-3-^{14}C had been released as $^{14}CO_2$ and 25% had been incorporated into cyst materials (Figure 1). Analyses of the various chemical fractions of the cyst by the method of Roodyn and Mandel (86) showed that 55%, 28%, and 21% of the total radioactivity incorporated into the cell were found, respectively, in the protein, lipid, and RNA fractions (Figure 2). No BHB-3-^{14}C was found in the DNA or acid-soluble fractions. In the RNA fraction, there was a steady increase in radioactivity until about the twelfth hour and then a slow decrease. The protein and lipid fractions showed a steady increase in the incorporation of BHB-3-^{14}C throughout encystment. The PHB content per cell increased 10-fold from 0.1 to 1 μg PHB per cell nitrogen at 30-33 hours, and then decreased to 0.8 μg PHB per μg cell nitrogen at the completion of encystment (Figure 3).

Addition of Cyclic AMP

The objective of the experiment was to test if cyclic AMP added to cultures of A. vinelandii could modify the rate of vegetative growth, overcome abortive encystment, or alter the rate of morphological development during encystment. At 12 hour intervals, the samples were examined under phase-contrast microscopy and compared to cells which had not received cyclic AMP. There were no morphological differences observed with cells cultivated in the presence or absence of the nucleotide. Vegetative,

Figure 1.--Distribution of BHB-3-^{14}C during encystment of A. vinelandii. BHB-3-^{14}C (0.5 μCi) and 0.2% unlabelled BHB were added to 100 ml Burk's nitrogen-free buffer to initiate encystment. Experimental details are reported in the text.

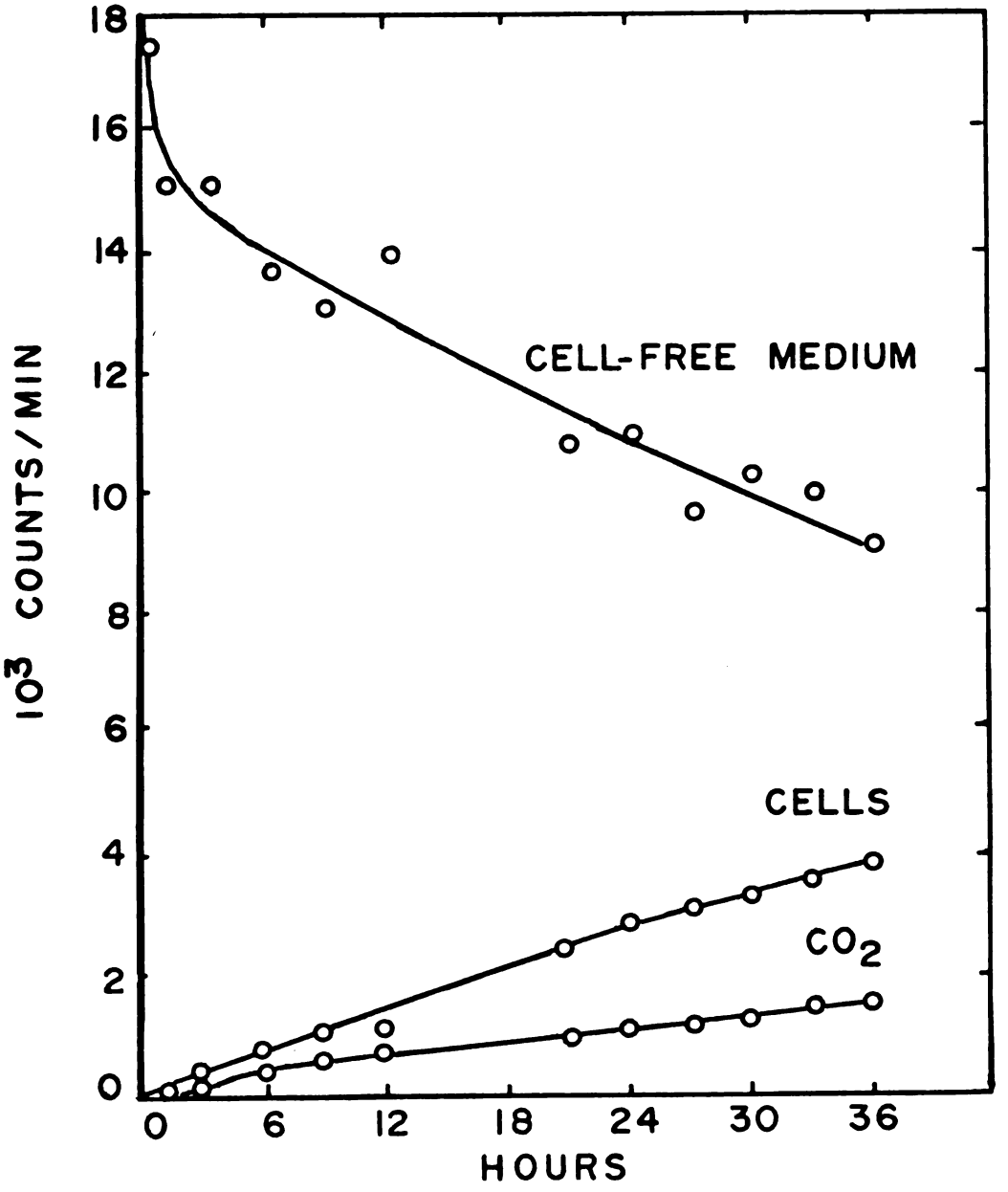

18
16
14
12
10
8
6
4
2
0
10^3 COUNTS/MIN
CELL-FREE MEDIUM
CELLS
CO_2
0
6
12
18
24
30
36
HOURS

Figure 2.--Distribution of BHB-3-^{14}C into cysts of A. vinelandii. BHB-3-^{14}C (10 μCi) and 0.2% unlabelled BHB were added to 100 ml Burk's nitrogen-free buffer to initiate encystment. See the text for experimental procedures.

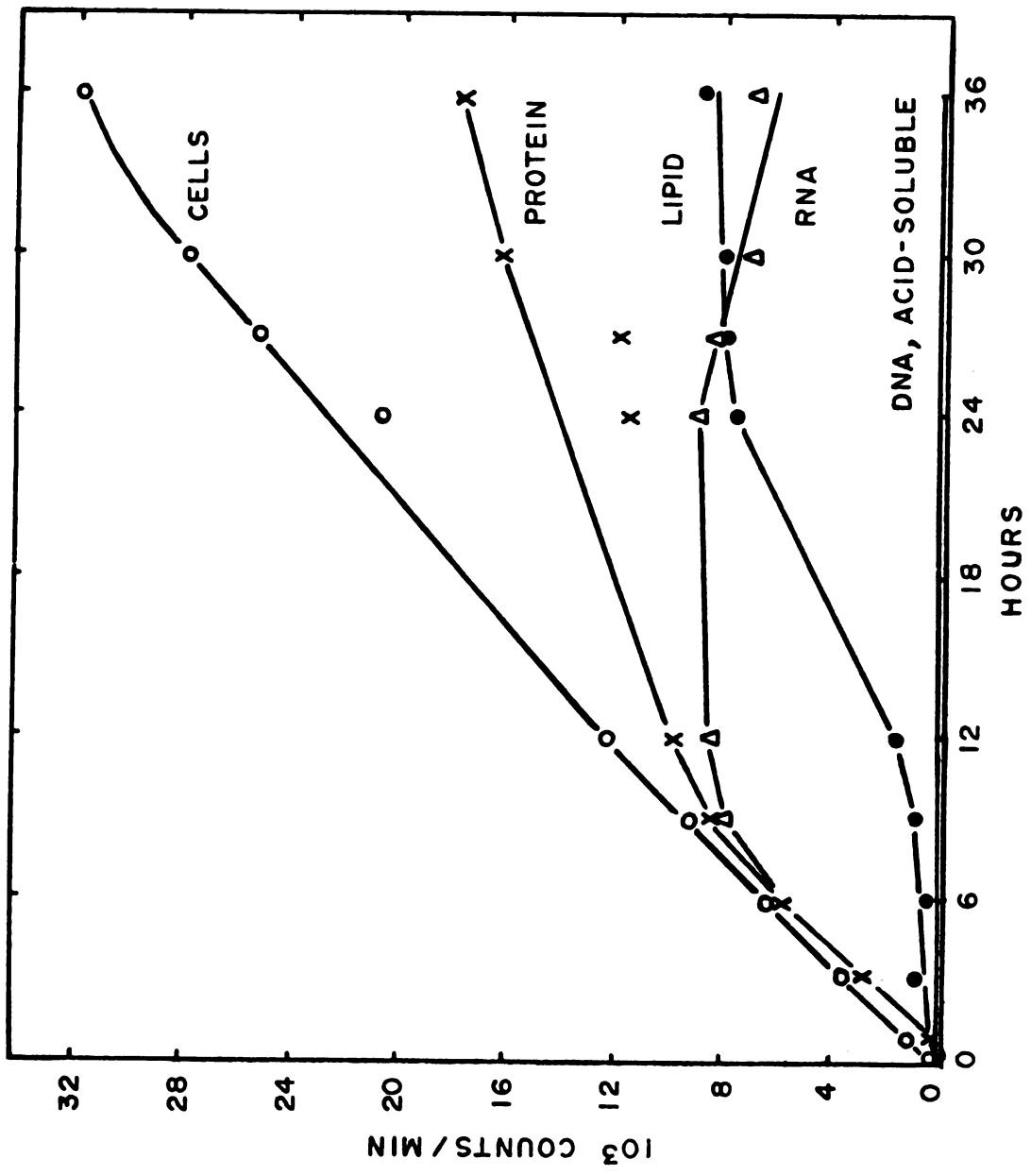
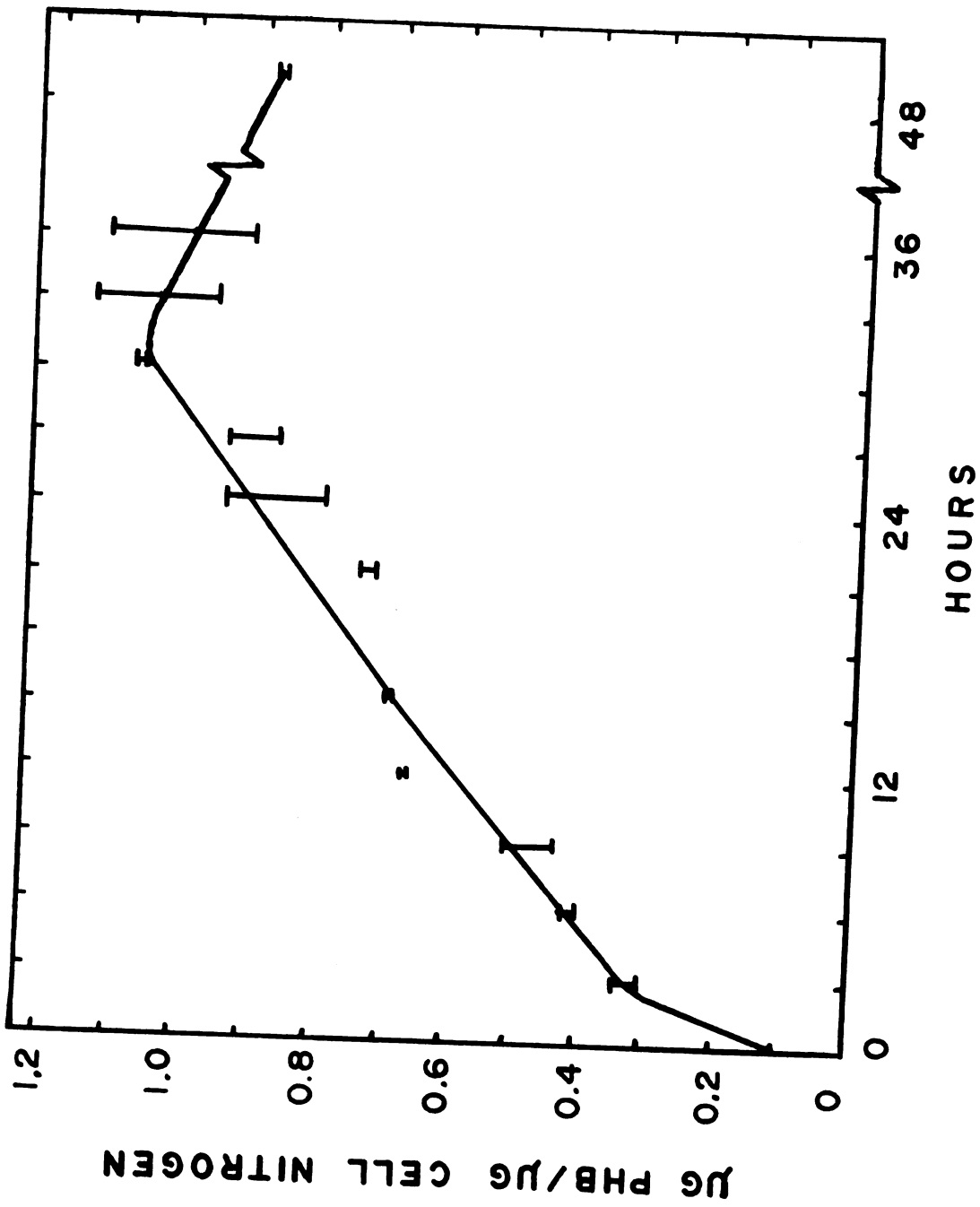

CELLS
PROTEIN
LIPID
RNA
DNA, ACID-SOLUBLE
HOURS
10³ COUNTS / MIN
0
4
8
12
16
20
24
28
32
0
6
12
18
24
30
36

Figure 3.--Poly-β-hydroxybutyrate content per μg cell nitrogen during encystment of A. vinelandii.

Cells were induced to encyst in 100 ml of Burk's buffer containing 0.2% BHB. At regular time intervals during the course of encystment, 1 ml cell samples were removed to determine the cell nitrogen content. At the same time, another set of 1 ml samples were removed and were placed in thick-walled glass centrifuge tubes containing 9 ml sodium hypochlorite. After 24 hours at 26 C, the cells were digested and the residue containing PHB was washed twice with distilled water, acetone, and diethyl ether. The polymer was dissolved in concentrated H_2SO_4 and read at 235 nm with a Perkin-Elmer double-beam spectrophotometer.


µG PHB/µG CELL NITROGEN
1.2
1.0
0.8
0.6
0.4
0.2
0
0
12
24
36
48
HOURS

encysting and abortively encysting cells were pretreated with Tris-EDTA for two minutes to increase their permeability to cyclic AMP (52, 78). These cells did not appear different from their respective untreated cells or cells that had not received cyclic AMP.

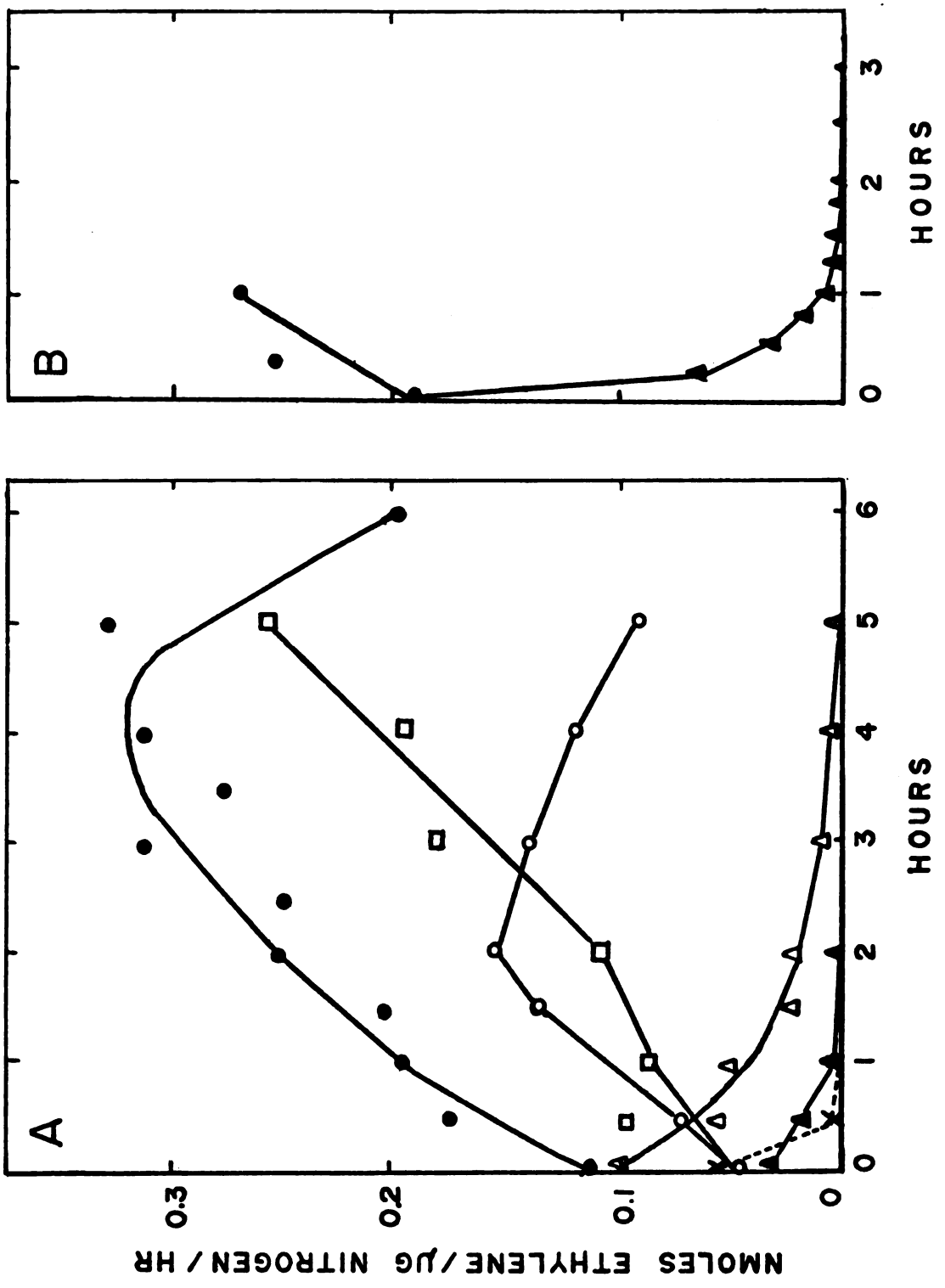
Nitrogen-Fixation

Nitrogen-fixation is a property of A. vinelandii cells which has been reported to be lost upon induction of encystment (89). The following experiments were carried out to determine the time course of nitrogenase loss and those conditions of encystment which contributed to the decrease in nitrogen-fixation.

Prior to the completion of exponential growth, vegetative cells were harvested and resuspended in fresh Burk's nitrogen-free buffer containing one of the following carbon sources: 1% glucose, 0.5% acetate, or 0.1% propanol for continued vegetative growth; and 0.2% BHB or 0.03% crotonate for encystment. The rate of acetylene reduction (nitrogen-fixation) of cells in glucose, acetate or propanol (vegetative growth) increased, reflecting the increase in cell mass. However, encysting cells lost their ability to reduce acetylene and within 15 minutes after the addition of BHB, the rate decreased by 70% and was approximately zero by the first hour (Figure 4).

Figure 4.--The rate of acetylene reduction by A. vinelandii in the presence of different carbon sources.

The rate of nitrogen fixation by A. vinelandii in the presence of different carbon sources (A), and the rate of nitrogen fixation of vegetative and encysting cells at 15 minute intervals (B) was measured by the procedure described in the text and plotted as nmoles of ethylene produced per µg cell nitrogen. Symbols: (●), 1% glucose (vegetative growth); (O), 0.5% acetate (vegetative growth); (□), 0.1% propanol (vegetative growth); (▲), 0.2% BHB (encystment); (Δ), 0.03% crotonate (encystment); (X), no substrate.


A
B
NMOLES ETHYLENE / µG NITROGEN / HR
0
0.1
0.2
0.3
0
1
2
3
4
5
6
HOURS
HOURS

The final cell division occurred 4-6 hours after the addition of BHB.

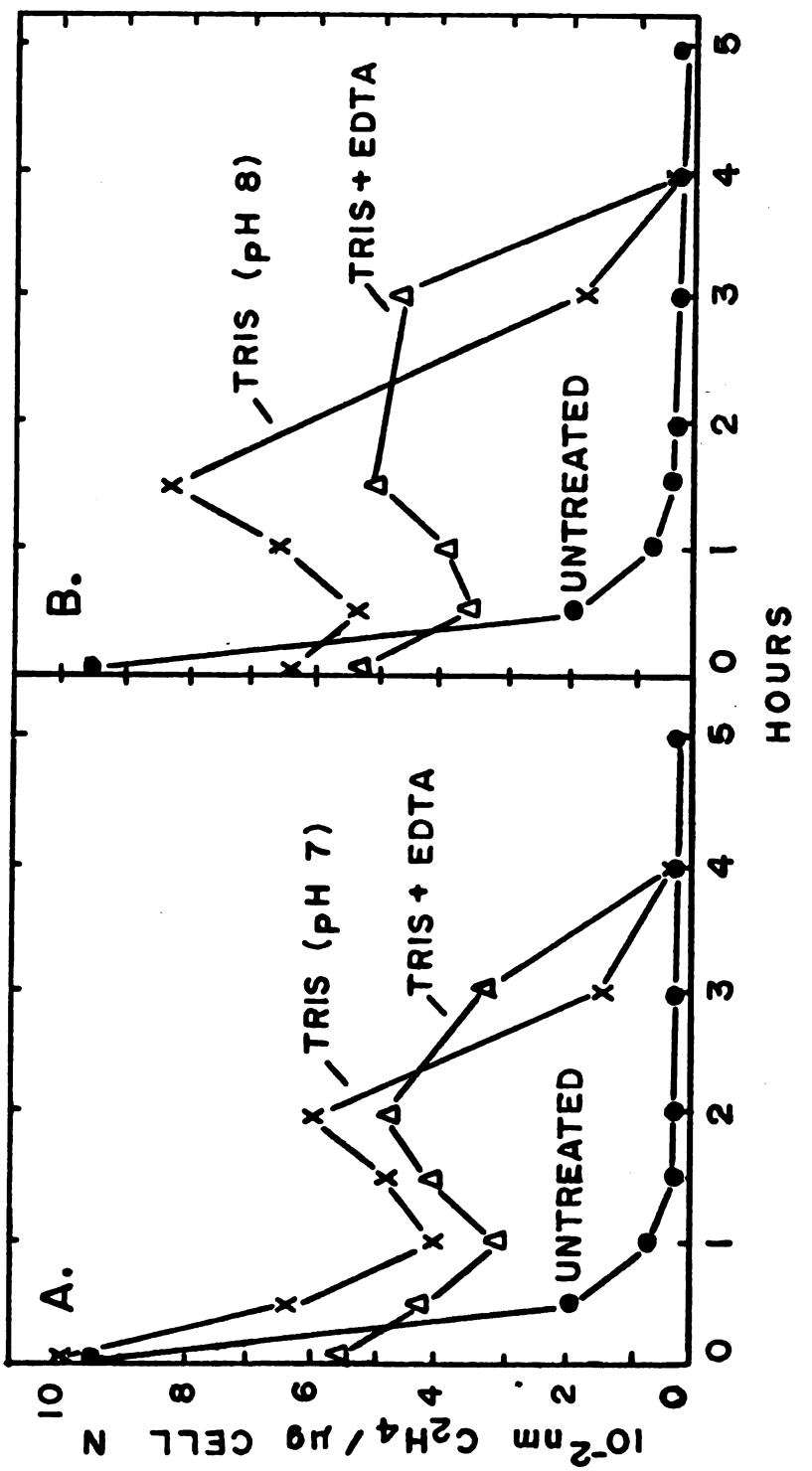
Cyclic AMP was added to exponentially growing and encysting cells to determine if nitrogen fixation was influenced by the concentration of the nucleotide in the cell. There were no observable differences in the amount of acetylene reduced in cells with or without exogenous cyclic AMP. However, encysting cells which were pretreated with Tris and/or EDTA showed a sudden drop in acetylene reduction during the first hour and then an increase of activity which peaked at the second hour, followed by a decrease again by the fourth hour (Figure 5). This alteration of acetylene reduction activity was also observed in encysting cells pretreated with Tris buffers at pH 7 and 8, with and without 10^{-3} M EDTA.

Enzyme Activities

The appearance (disappearance) and time course for the induction of key enzymes in the Entner-Doudoroff pathway, TCA cycle, glyoxylate shunt and gluconeogenesis were assayed in A. vinelandii to aid in determining the role and fate of BHB during encystment. During a shift from carbohydrate (glucose) to lipid (BHB) metabolism, the induction of the enzymes of the glyoxylate shunt and gluconeogenesis would enable the cells to conserve carbon atoms and to convert BHB into the cyst coat materials.

Figure 5.--The rate of acetylene reduction of encysting cells pretreated with Tris with and without EDTA.

Vegetative cells of A. vinelandii were harvested, washed and pretreated with 0.12 M Tris-glycerol-phosphate buffer, pH 7 (A) or pH 8 (B), and without or with 10^{-3} M EDTA for two minutes. These cells were then induced to encyst in the presence of 0.2% BHB and the rate of nitrogen fixation was followed by the experimental procedure described in the text and plotted as nmoles of ethylene produced per µg cell nitrogen. Symbols: (X), without EDTA; (Δ), with 10^{-3} M EDTA; (●), untreated cells.


A.
B.
TRIS (pH 7)
TRIS + EDTA
UNTREATED
TRIS (pH 8)
TRIS + EDTA
UNTREATED
HOURS
10^{-2} nm C_2H_4 / µg CELL N
0
2
4
6
8
10
0
1
2
3
4
5

Encystment was characterized by the steady decrease in specific activity of glucose-6-phosphate dehydrogenase, a key enzyme of the Entner-Doudoroff and the pentose phosphate pathways of glucose catabolism in vegetative cells of A. vinelandii (66, 99) (Figure 6). Also shown in Figure 6 are the activities of BHB dehydrogenase and isocitrate dehydrogenase which each exhibited two peaks of activity at 6 and 21 hours. The addition of both glucose and BHB (induction of abortive encystment) to washed, exponentially growing cells resulted in a three hour time lag in appearance of BHB dehydrogenase and an approximately 25% decrease in its activity (Figure 7).

Isocitrate lyase activity was very low in vegetative cells which had been grown on glucose. However, upon the replacement of glucose with BHB in the vegetative cells, there was an immediate induction of the enzyme which then decreased to the low initial value by 9 hours. The enzyme was again induced at 15 hours and achieved high levels of activity in cells late in the encystment period. The activity of malate synthase appeared to be coordinated with that of isocitrate lyase (Figure 8).

The two enzymes of gluconeogenesis, aldolase and FDPase, which appear during encystment have a temporal relationship (Figure 9). Aldolase activity had a maximal value at 6 hours and a minor one at 24 hours. FDPase had

Figure 6.--The time course for the appearance of glucose-6-phosphate dehydrogenase, BHB dehydrogenase, and isocitrate dehydrogenase during encystment of A. vinelandii.

The procedure for preparing cell-free extracts and assaying for enzyme activities are described in the text. Symbols: (●), glucose-6-phosphate dehydrogenase; (Δ), BHB dehydrogenase; (X), isocitrate dehydrogenase.

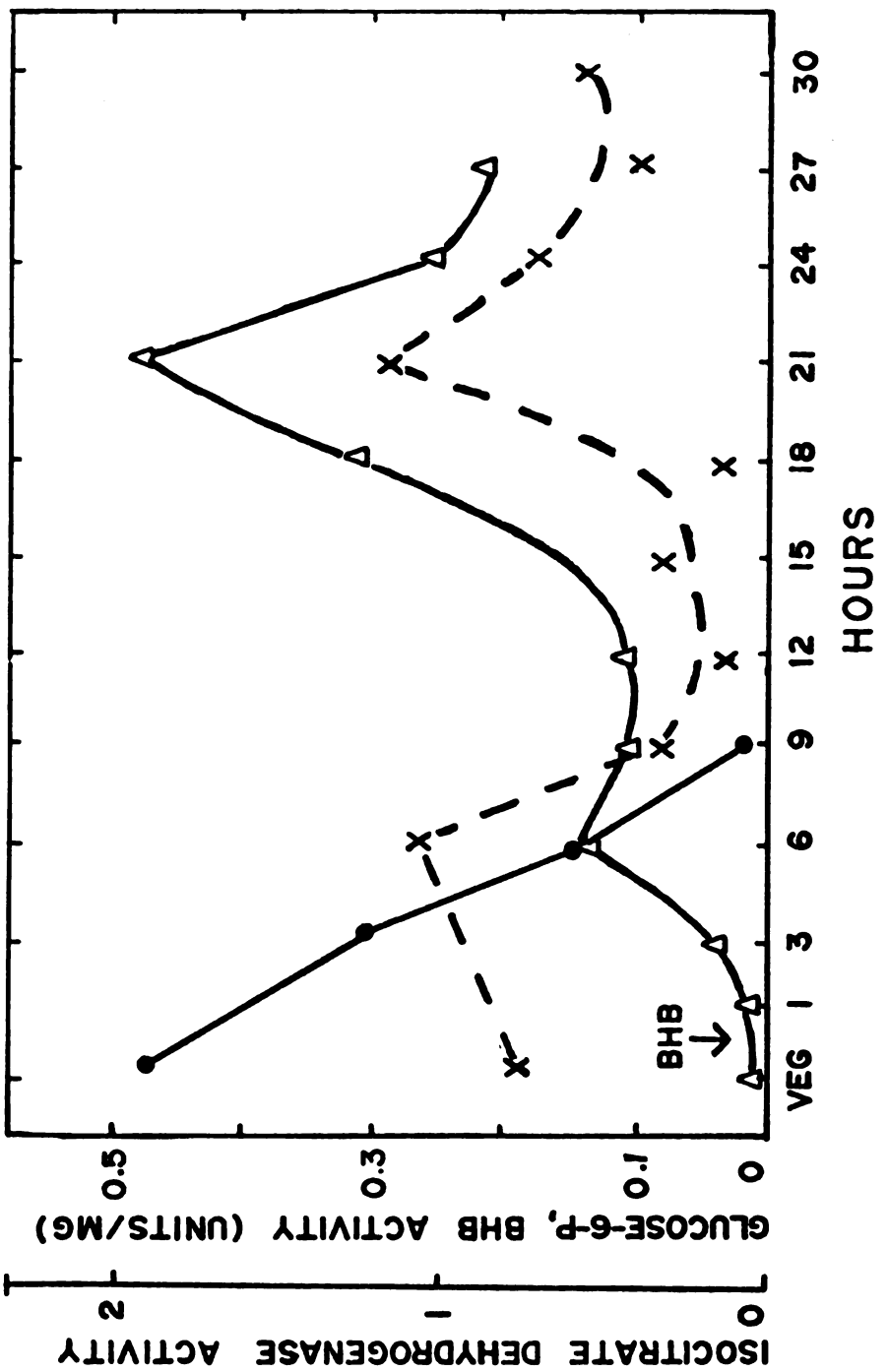

ISOCITRATE DEHYDROGENASE ACTIVITY
0
1
2
GLUCOSE-6-P, BHB ACTIVITY (UNITS/MG)
0
0.1
0.3
0.5
VEG
1
3
6
9
12
15
18
21
24
27
30
HOURS
BHB

Figure 7.--The time course for the appearance of BHB dehydrogenase during encystment and abortive encystment.

The procedure for preparing cell-free extracts and assaying for enzyme activities are described in the text. Symbols: (●), encystment (0.2% BHB); (O), abortive encystment (0.2% glucose and 0.2% BHB).

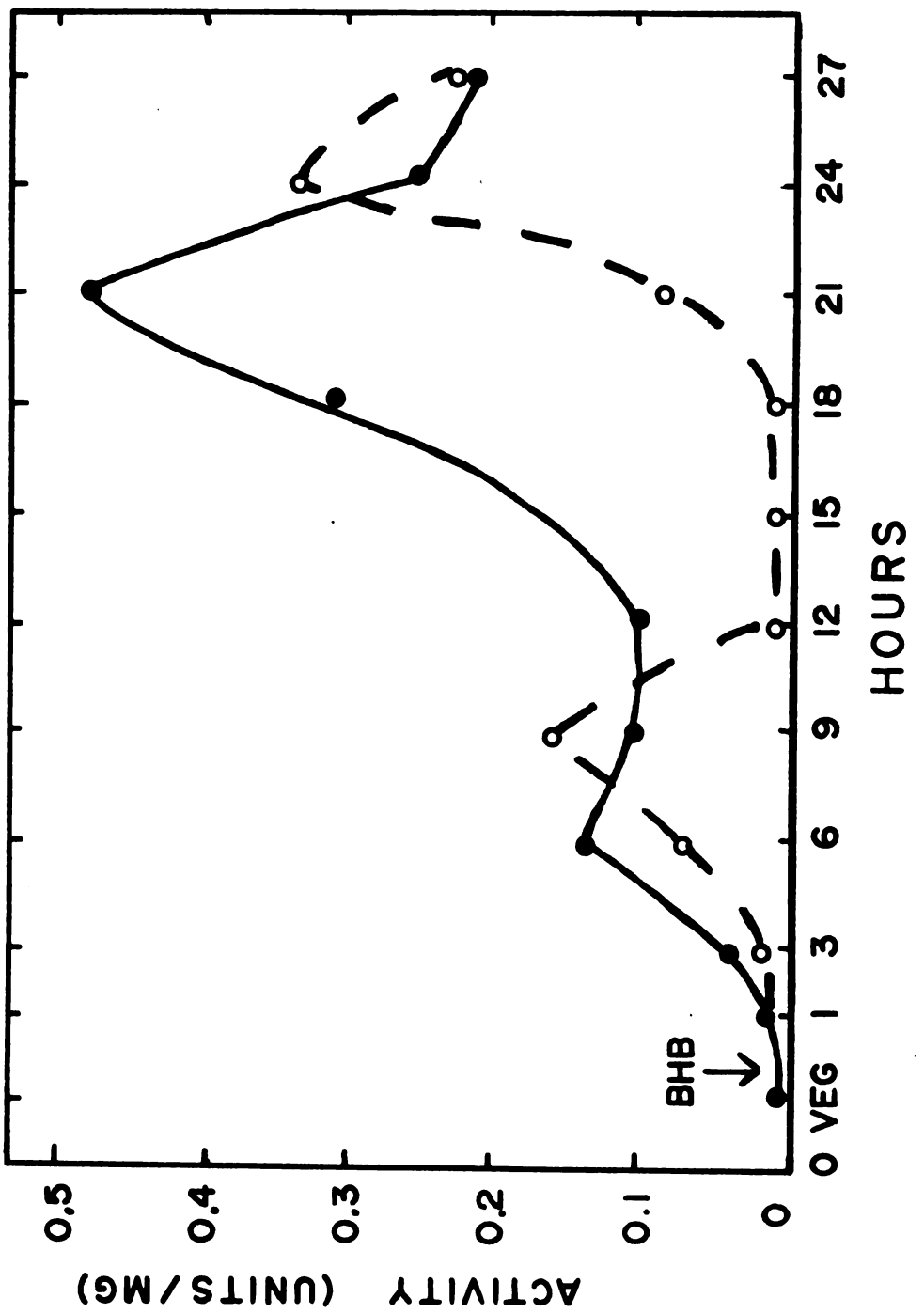

ACTIVITY (UNITS/MG)
0.5
0.4
0.3
0.2
0.1
0
0 VEG
1
3
6
9
12
15
18
21
24
27
HOURS
BHB

Figure 8.--The time course for the appearance of the two glyoxylate shunt enzymes during encystment.

See text for experimental procedures. Symbols: (●), isocitrate lyase; (O), malate synthase.

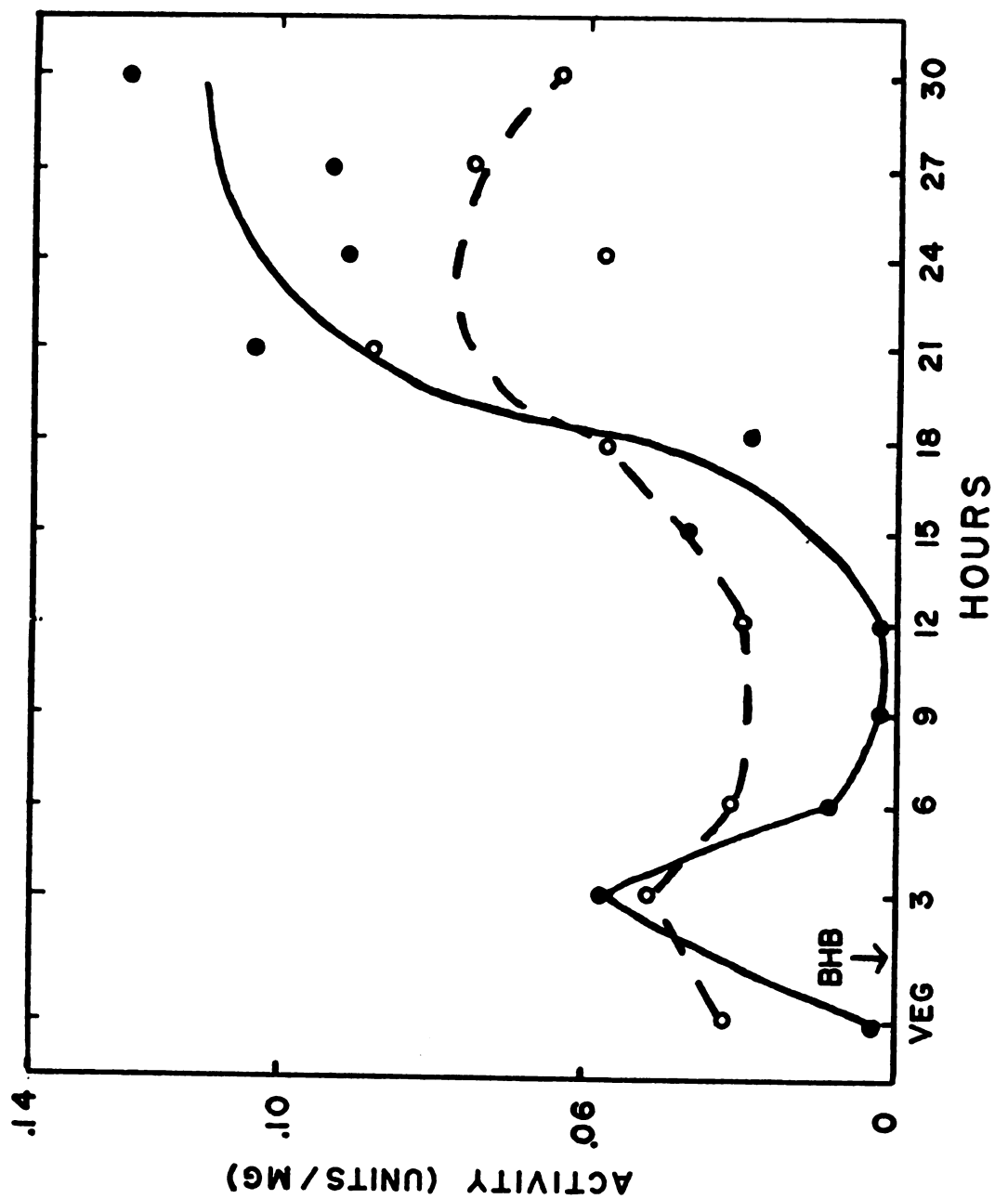
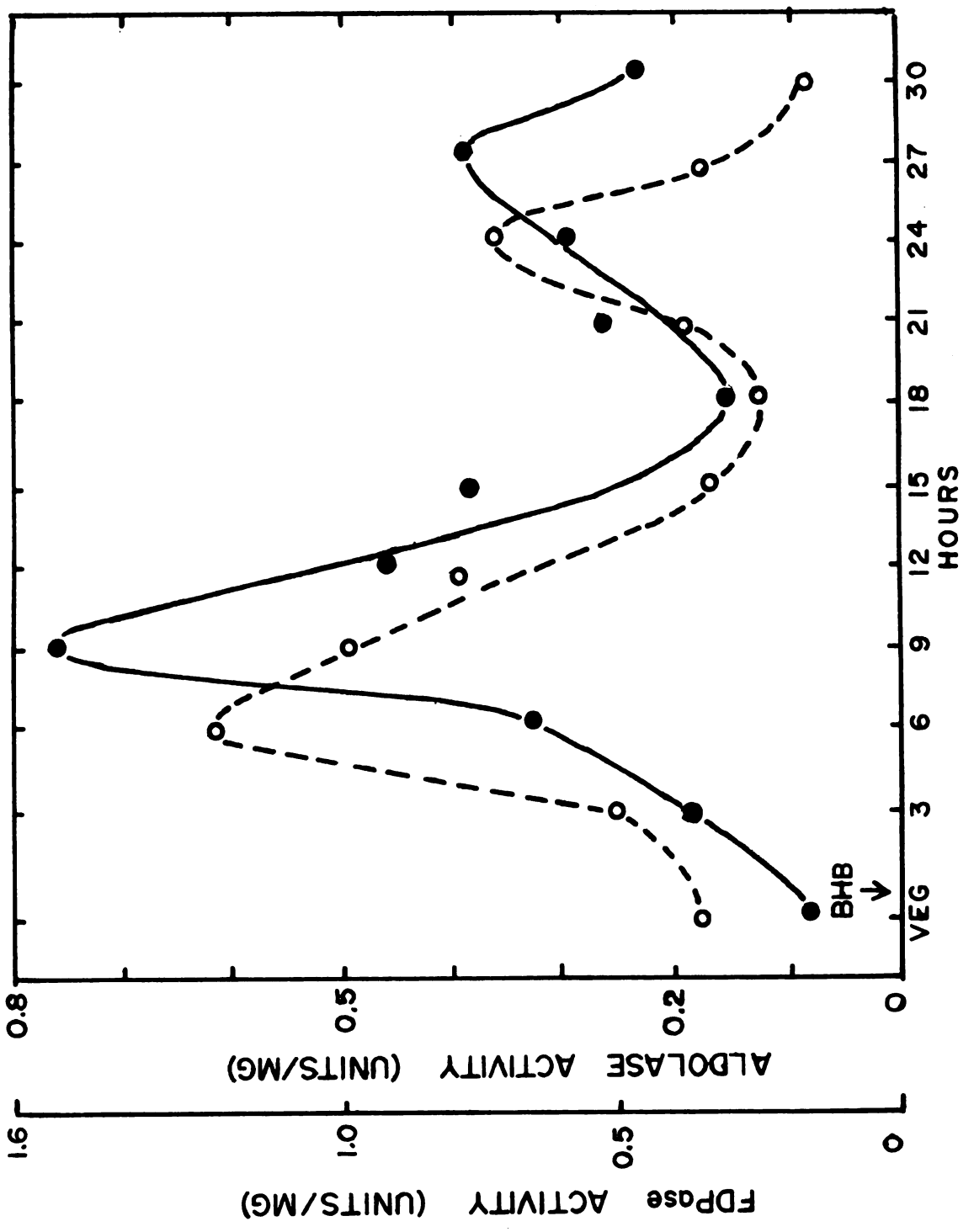

ACTIVITY (UNITS/MG)
.14
.10
.06
0
VEG
BHB
3
6
9
12
15
18
21
24
27
30
HOURS

Figure 9.--The time course for the occurrence of the two enzymes of gluconeogenesis, aldolase and FDPase, which appear during encystment.

The experimental details are reported in the text. Symbols: (O), aldolase; (●), FDPase.


FDPase ACTIVITY (UNITS/MG)
1.6
1.0
0.5
0
ALDOLASE ACTIVITY (UNITS/MG)
0.8
0.5
0.2
0
VEG
BHB
3
6
9
12
15
18
21
24
27
30
HOURS

two peak activity periods in encysting cells, at 9 and again at 27 hours.

Macromolecular Synthesis

The previous experiments showed that BHB was incorporated into A. vinelandii cells and it induced increased activities of the key enzymes in the TCA cycle, glyoxylate shunt and gluconeogenesis. The macromolecular synthesis associated with cyst formation were also examined to elucidate further the role of BHB during encystment.

Upon the addition of BHB to the cells to induce encystment, the rate of DNA synthesis began to cease at about the fourth hour just prior to the final cell division which resulted in the formation of the precyst (28) (Figure 10). On the other hand, net RNA synthesis continued until the twelfth hour of encystment, although at a lower rate than in exponentially growing cells. Protein synthesis, measured as ^{14}C-leucine incorporation, continued throughout the 36-hour encystment period but at approximately one-third the rate which was occurring in vegetative cells (Figure 11).

In an attempt to establish a control during protein synthesis, it was discovered that encysting A. vinelandii cells were very resistant to chloramphenicol. Protein synthesis and the rate of growth of exponential cultures of A. vinelandii became progressively more inhibited by increasing concentrations of chloramphenicol.

Figure 10.--DNA and RNA synthesis by cells of A. vinelandii during vegetative growth and encystment.

Cells of A. vinelandii were grown to the end of exponential phase in 1% glucose, harvested and washed with fresh Burk's buffer. One-half of the cells continued vegetative growth in 100 ml fresh Burk's buffer containing 1% glucose; uracil-2-^{14}C (21.5 µCi) and 45 µmole uracil for DNA synthesis, or uracil-2-^{14}C (10 µCi) and 20 µg cytosine per ml for RNA synthesis. The other half of the cells were induced to encyst in 100 ml fresh Burk's buffer containing 0.2% BHB; uracil-2-^{14}C (21.5 µCi) and 45 µmole uracil for DNA synthesis, or uracil-2-^{14}C (10 µCi) and 20 µg cytosine per ml for RNA synthesis. See the text for experimental procedures for determining DNA and RNA synthesis. Symbols: (circles), DNA; (triangles), RNA; (closed symbols), vegetative growth; (open symbols), encystment.

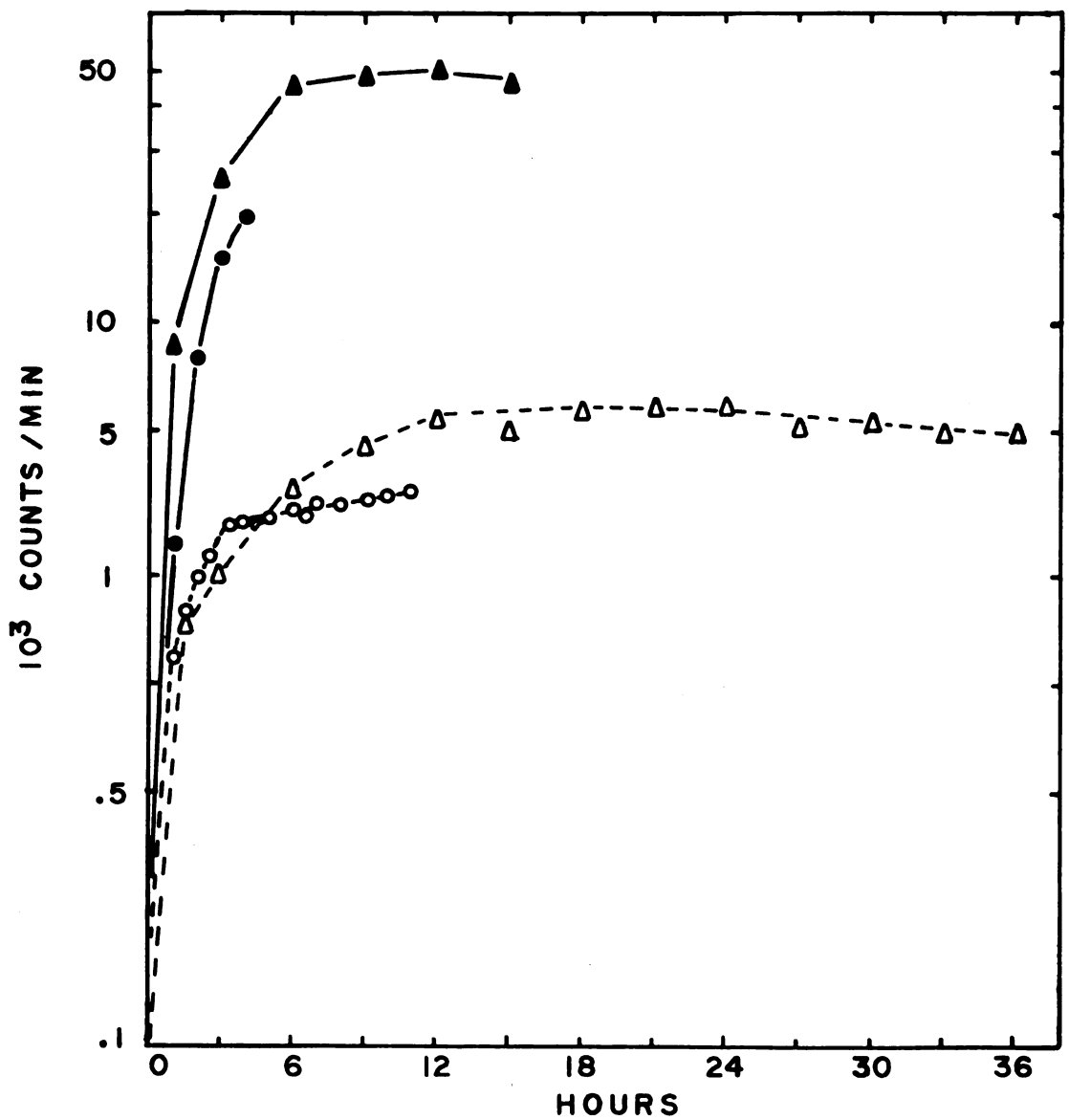
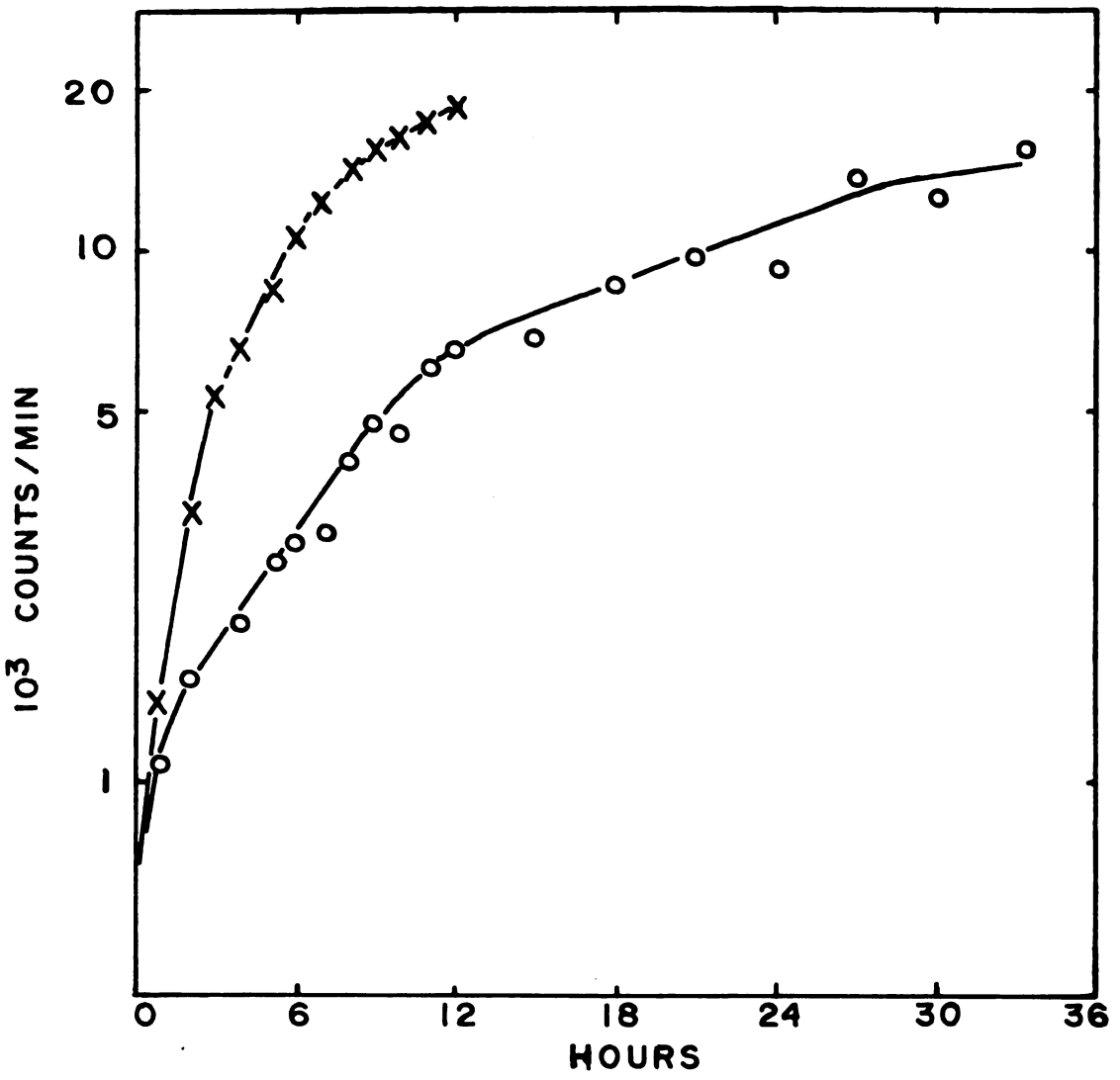

50
10
5
1
.5
.1
10^3 COUNTS / MIN
0
6
12
18
24
30
36
HOURS

Figure 11. Protein synthesis during vegetative growth and encystment.

Cells of A. vinelandii were grown to the end of exponential phase in 1% glucose, harvested and washed. One-half of the cells were induced to encyst in 100 ml Burk's buffer containing 0.2% BHB, L-leucine-U-^{14}C (10 µCi) and 5 µmole L-leucine per ml, while the other half continued vegetative growth in 100 ml fresh Burk's buffer containing 1% glucose, L-leucine-U-^{14}C (10 µCi) and 5 µmole L-leucine per ml. The experimental procedures for determining ^{14}C-leucine incorporation into cells are given in the text. Symbols: (X), vegetative growth; (O), encystment.


20
10
5
1
10^3 COUNTS/MIN
0
6
12
18
24
30
36
HOURS

Concentrations of 75 and 100 µg of the antibiotic per ml reduced ^{14}C-leucine uptake in cells by 85 and 90%, respectively (Figure 12). The growth rates of cells utilizing 0.1% propanol or 0.5% acetate as substrate were inhibited 60 and 67%, respectively, by the addition of 50 µg of chloramphenicol per ml (Figure 13). However, immediately upon the induction of encystment with BHB, cells of A. vinelandii became resistant to chloramphenicol at concentrations up to 100 µg per ml. The rate of protein synthesis by these cells was reduced by 25% in the presence of 100 µg chloramphenicol per ml but morphogenesis was not interrupted (Figure 14). The usual sequence of events which were discernible by phase-contrast microscopy was identical in encysting cells with or without chloramphenicol. Cells pretreated with Tris-EDTA also displayed similar resistance to the antibiotic during encystment.

The distribution of L-leucine-U-^{14}C into various chemical fractions of encysting cells in the presence or absence of 100 µg chloramphenicol per ml was studied by the method of Roodyn and Mandel (86) to see if the leucine was being incorporated into proteins or into other cellular macromolecules under the influence of the antibiotic. The results (Figure 15) showed that during encystment 90-100% of the ^{14}C-leucine was found in the protein fraction. There was no ^{14}C-leucine found in the CNA and acid-soluble

Figure 12.--The growth of cells and the extent of protein synthesis of vegetative cells in the absence or presence of chloramphenicol.

The rate of growth of vegetative cells of A. vinelandii was monitored by following the turbidity at 600 nm (A), and protein synthesis was determined by the method described in the text. Symbols: (O), without chloramphenicol (CM); (Δ), 75 μg CM/ml; (X), 100 μg CM/ml; (●), 150 μg CM/ml.

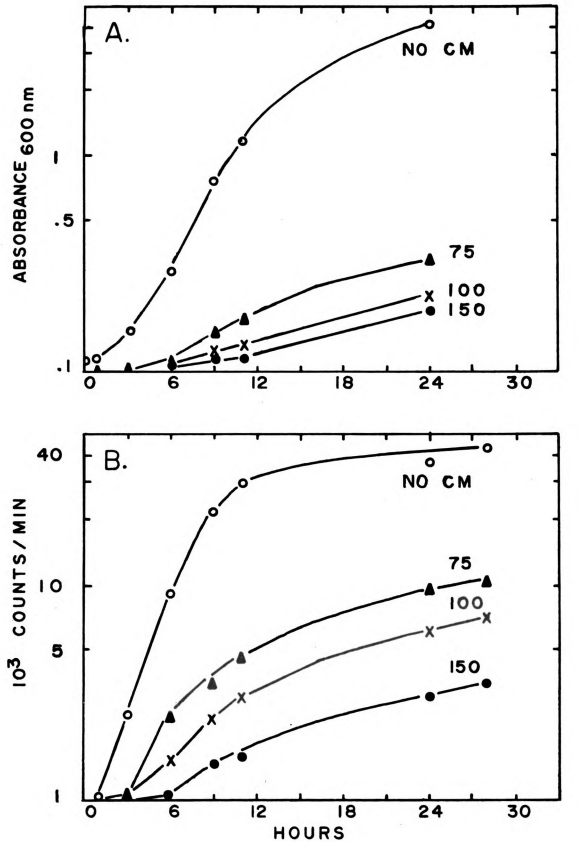

A.
NO CM
75
100
150
ABSORBANCE 600 nm
1
.5
.1
0
6
12
18
24
30
B.
NO CM
75
100
150
40
10
5
1
10^3 COUNTS / MIN
0
6
12
18
24
30
HOURS

Figure 13.--The growth of vegetative cells in the presence of 0.5% acetate or 0.1% propanol with and without chloramphenicol.

Vegetative cells of A. vinelandii were cultivated in 0.5% acetate (A) and 0.1% propanol (B), and the rate of growth in the absence or presence of chloramphenicol was monitored by measuring the turbidity at 600 nm. Symbols: (X), without chloramphenicol (CM); (Δ), 50 μg CM/ml; (●), 100 μg CM/ml; (O), vegetative cells in 1% glucose.

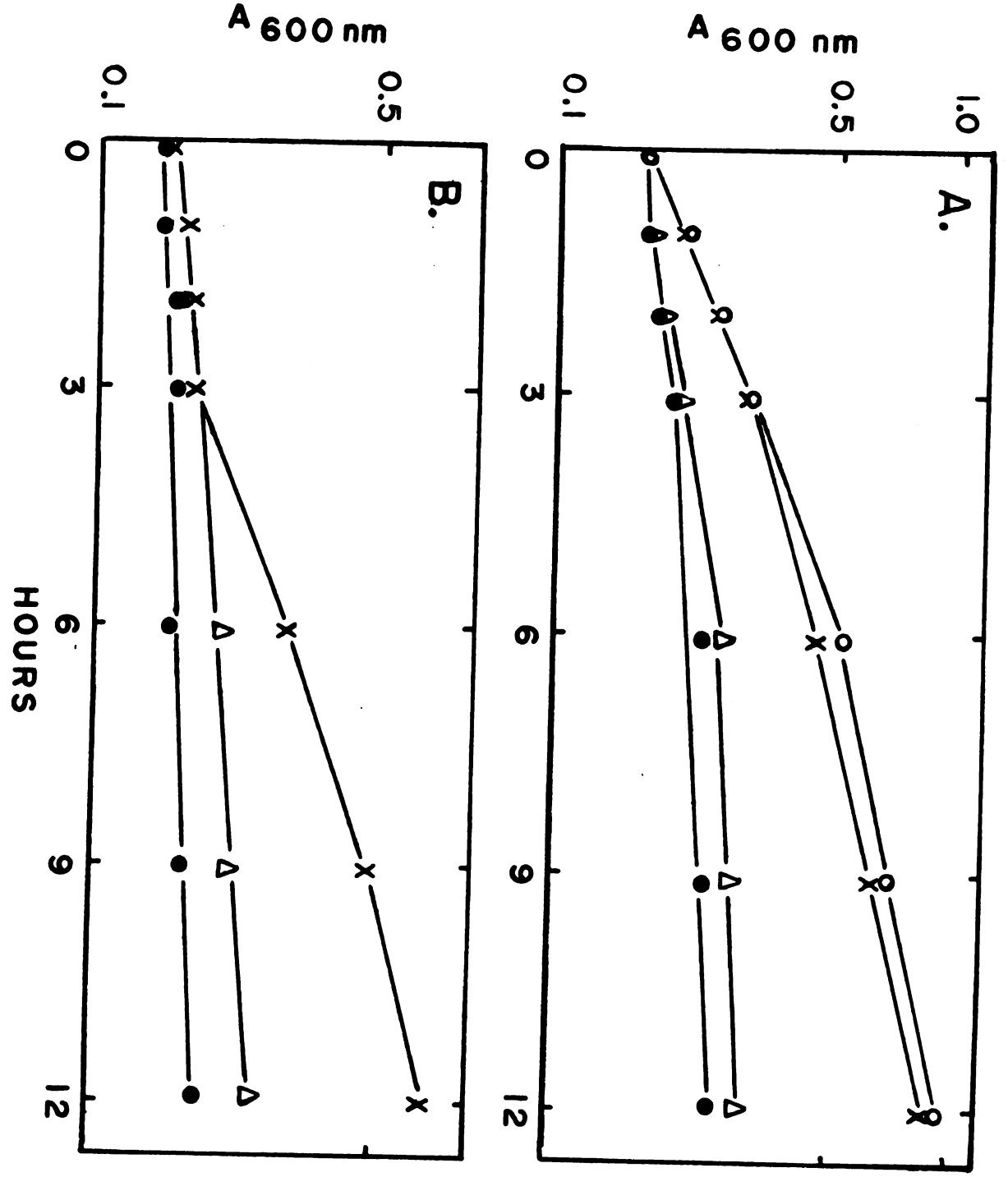

A 600 nm
0.1
0.5
1.0
A.
B.
0
3
6
9
12
HOURS

Figure 14.--The growth of cells and the extent of protein synthesis of encysting cells of A. vinelandii in the absence or presence of chloramphenicol.

Vegetative cells at the end of exponential growth were harvested, washed and equally divided into three flasks each containing 100 ml fresh Burk's buffer, 0.2% BHB, L-leucine-U-^{14}C (5 µCi) and 5 µmole L-leucine per ml. Two flasks contained different concentrations of chloramphenicol. The rate of growth (A), and protein synthesis (B) were determined by the experimental procedures described in the text. Symbols: (O), without chloramphenicol (CM); (Δ), 75 µg CM/ml; (X), 100 µg CM/ml.

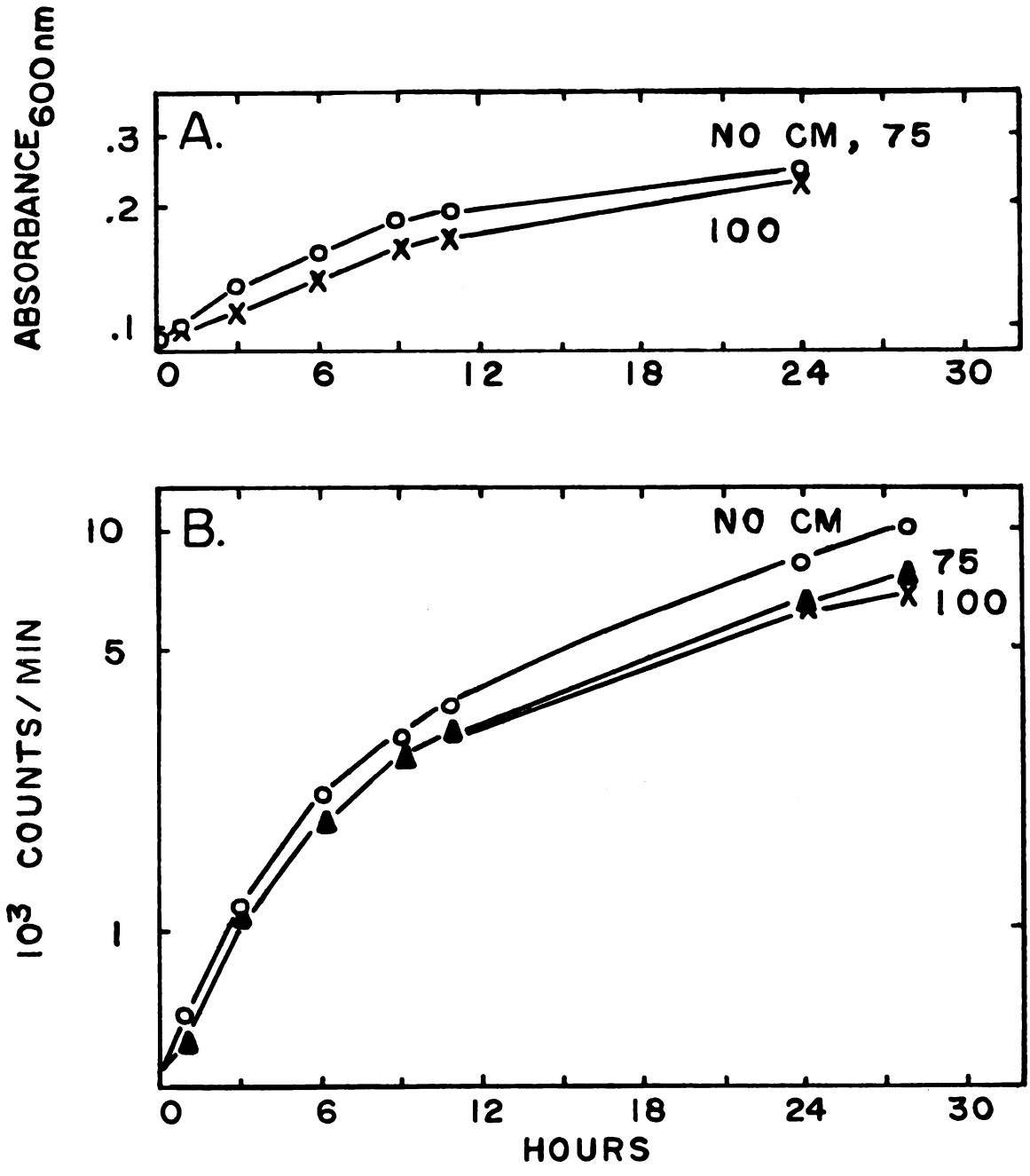
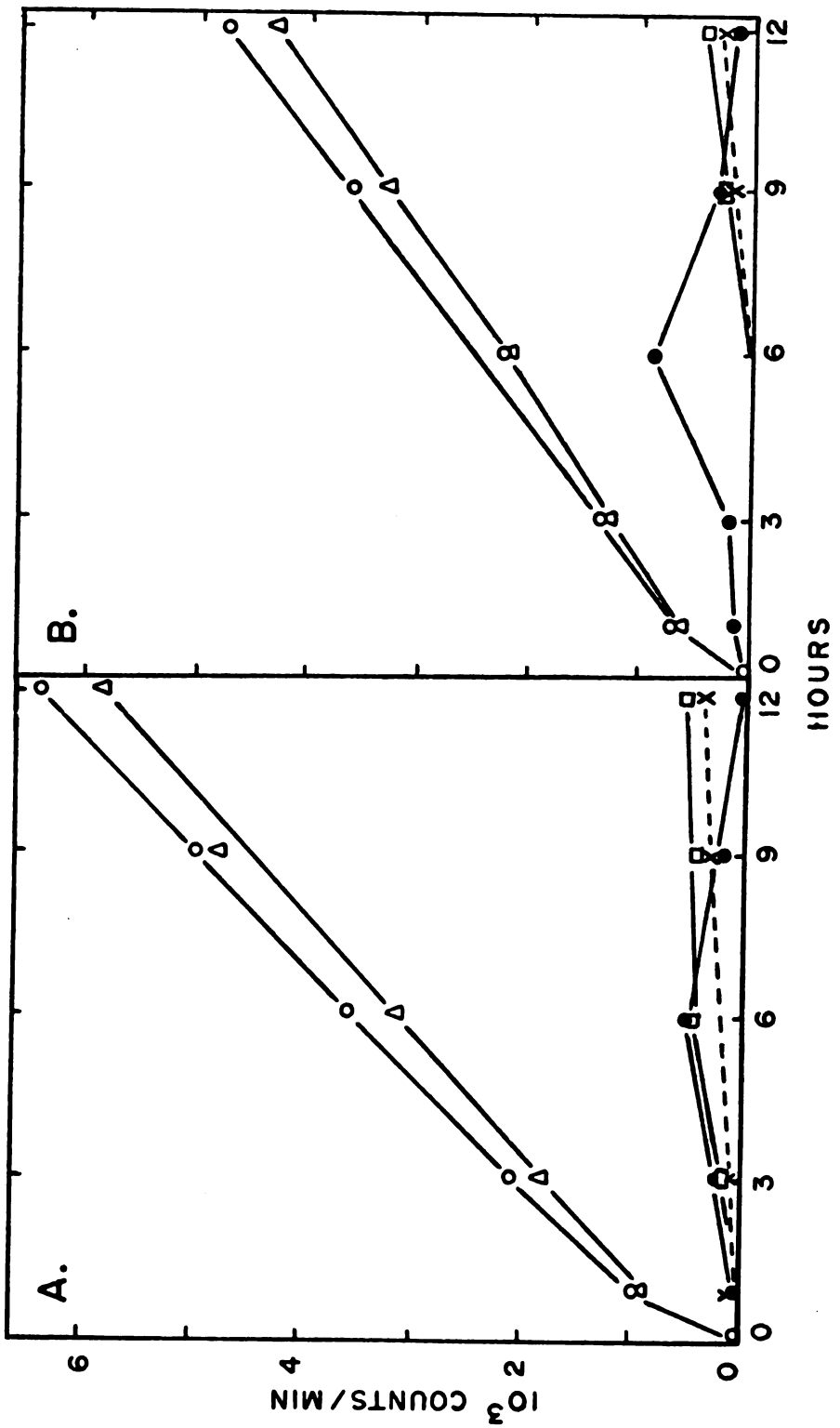

ABSORBANCE 600 nm
.3
.2
.1
A.
NO CM, 75
100
0
6
12
18
24
30
10
5
1
10^3 COUNTS/MIN
B.
NO CM
75
100
HOURS

Figure 15.--The distribution of L-leucine-U-^{14}C into various fractions of encysting cells of A. vinelandii.

Cells were induced to encyst in 100 ml Burk's buffer containing 0.2% BHB, L-leucine-U-^{14}C (5 μCi) and 5 μmole L-leucine per ml in the absence (A), or presence of 100 g chloramphenicol per ml (B). See text for experimental procedures. Symbols: (O), whole cells; (Δ), protein; (□), RNA; (●), acid-soluble; (X), lipid.


A.
B.
10^3 COUNTS / MIN
HOURS
0
2
4
6
0
3
6
9
12

fractions although small quantities were noted in the lipid and RNA fractions.

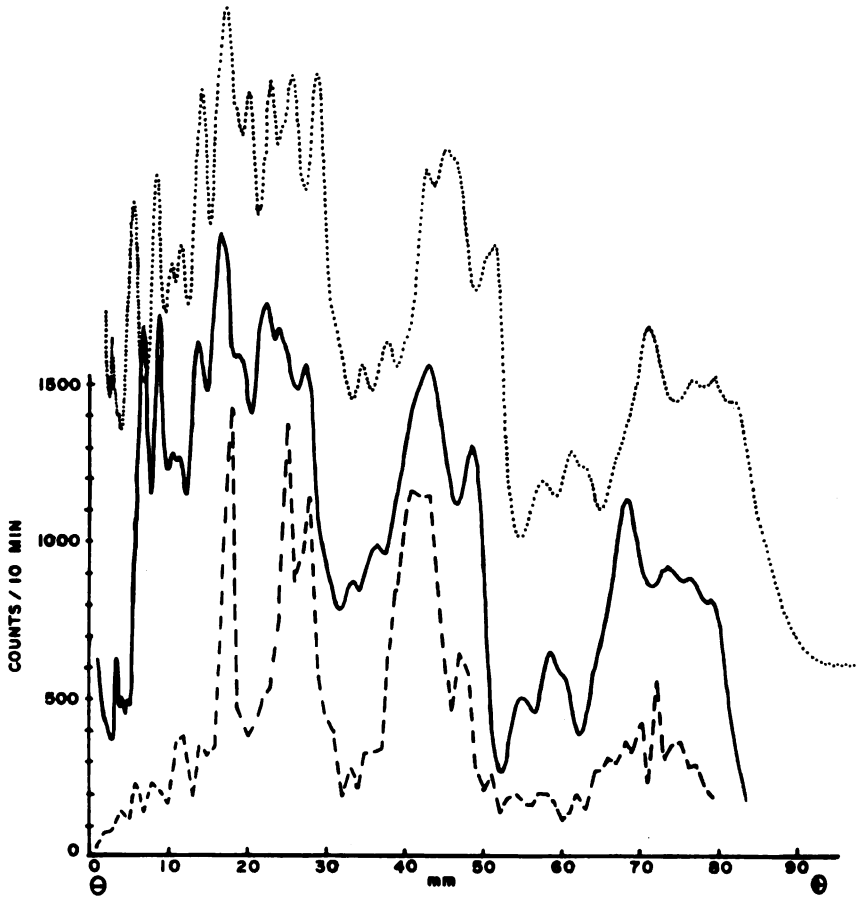
The chemical fractionation method of Roodyn and Mandel (86) does not differentiate between normal protein synthesis (messenger RNA-directed) or ^{14}C-leucine incorporation into polypeptides whose synthesis could be enzyme-directed. The striking difference between these two classes of peptides would be in their sizes. Therefore proteins made during encystment in the presence or absence of chloramphenicol were solubilized and analyzed by SDS-gel electrophoresis. The protein subunits were stained, and counted to observe the nature of the proteins which were synthesized during encystment. The pattern of distribution of radioactivity in the ^{14}C-leucine SDS-protein subunits correlated well with the gel scans of total protein subunits stained with Coomassie brilliant blue (Figure 16). In addition, the gel scans of protein subunits from encysting cells grown in the presence or absence of chloramphenicol were qualitatively similar; each class exhibited the same number of protein peaks.

Phospholipid Analysis

The dramatic acquisition of chloramphenicol resistance suggested that immediately upon the initiation of encystment a modification occurred in the permeability of the membrane to the antibiotic. This type of event could be produced by the formation and incorporation of a

Figure 16.--Analysis of SDS-polyacrylamide gels of protein subunits from encysting cells.

SDS-protein (100 µg protein per gel) from cells encysting in 50 ml Burk's buffer containing 0.2% BHB, L-leucine-U-^{14}C (5 µCi) and 5 µmole L-leucine per ml were separated into subunits by SDS-gel electrophoresis. One set of gels was stained with Coomassie brilliant blue and scanned at 550 nm with a Gilford spectrophotometer (dotted line) while the other set was sliced into 1 mm thick slices and counted for radioactivity (solid line). Similarly, a culture of cells encysting in the presence of 100 µg chloramphenicol per ml was treated as described above and the distribution of radioactivity in the SDS-protein subunits was determined (dashed line).


COUNTS / 10 MIN
1500
1000
500
0
0
10
20
30
40
50
60
70
80
90
mm
Θ
Θ

modified phospholipid into the cell membrane. The cessation of nitrogen fixation already noted is also consistent with such an event. Therefore the possibility of incorporation of BHB into membranes was investigated.

Within one-half hour after the induction of encystment, approximately 45% of the total BHB-3-^{14}C incorporated into the cell up to that time was located in the phospholipid fraction of the encysting cell membrane. Although the amount of BHB incorporated into the phospholipids increased during the course of encystment, it was not proportional to the increase taken up by the whole cell. Thus the percentage of BHB-3-^{14}C incorporated into the phospholipid fraction versus the total radioactive BHB incorporated into the entire cell decreased from 45% at 0.5 hour to 2.2% at 6 hours (Table 1).

Four kinds of phospholipids could be detected with the mercury molybdate reagent on the thin-layer chromatographic plates. One of these phospholipids was also ninhydrin positive, suggesting that it was phosphatidyl ethanolamine, the principal phospholipid of Azotobacter cells (42, 45, 83). All four phospholipids were radioactive, and the majority of the BHB-3-^{14}C was found in the phosphatidyl ethanolamine (Figure 17).

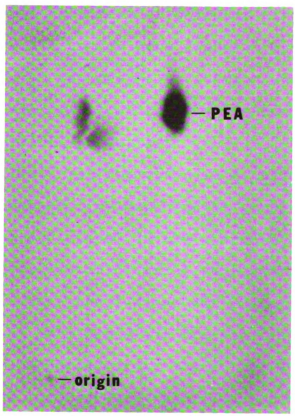
TABLE 1.--Incorporation of BHB-3-^{14}C into the phospholipid fractions during encystment.

Time of Sample	Counts per Minute		$\frac{\text{Cpm phospholipid}}{\text{Cpm cells}} \times 100$[a]
	50 ul phospholipid	0.2 g cells	
0.5 hr	10,980	29,300	45.1%
	13,590	29,300	
	16,340	31,520	
1.0 hr	6,435	41,300	16.2%
	6,400	37,480	
2.0 hr	15,385	134,000	10.7%
	13,200	132,740	
3.0 hr	4,850	100,890	4.2%
	2,500	61,880	
6.0 hr	4,030	201,465	2.2%
	4,250	177,500	

[a]See text for experimental details.

Figure 17.--Autoradiogram of thin-layer chromatography of phospholipids of 0.5-hour old encysting cells.

See text for experimental procedures for the extraction and separation of phospholipids. Two-dimensional chromatography of 20 µl of phospholipid extract labelled with BHB-3-^{14}C was applied at the origin on a Silica Gel G thin-layer plate and developed in the first direction (vertical) in the chloroform-methanol-acetic acid-water (100:50:14:6) solvent and in the second direction (horizontal) in the diisobutylketone-acetic acid-water (12:5:1) solvent. Symbol: PEA = phosphatidyl ethanolamine.


PEA
origin

DISCUSSION

Encystment is a form of cellular differentiation which requires differential gene expression. This results in the induction of new enzyme activities whose consequence is the development of a cell with new morphology and modified physical properties.

For optimal cyst formation in A. vinelandii, the cells must be removed from exponential growth conditions (glucose medium) and placed in the presence of BHB or one of its derivatives. Studies using radioactive-labelled BHB demonstrated that the inducer BHB functions as an energy source and as a precursor for the synthesis of cyst components. Approximately 25% of the radioactive-labelled BHB was incorporated into the cysts and another 10% converted to $^{14}CO_2$. Presumably the oxidation of BHB during encystment was well coupled to phosphorylation as was the oxidation of acetate during exponential growth of the organism. Chemical fractionation of the cysts revealed that BHB-3-^{14}C was located in the RNA, protein and lipid fractions. The latter two fractions were presumably in the exine and intine coats.

The chemical fractionation method of Roodyn and Mandel (86) is simple but limited in its applicability.

It does not permit the determination of radioactivity in the carbohydrate fractions which could be associated with the cell wall or cyst coats. A portion of the radioactivity associated with the RNA, lipid and protein fractions may also represent carbohydrate material (86). However, the polysaccharide content in the cell may be small, and is estimated to be less than 2% (85). A more laborious method of total chemical extraction and analysis of cysts would have permitted greater accuracy for determining the distribution of BHB into cyst components.

The repression of cyst formation by glucose appeared to be similar to the widely observed phenomenon of catabolite repression which can be overcome by increasing the intracellular levels of cyclic AMP (77). Cyclic AMP was added to abortive encysting cells in an attempt to overcome repression by 0.1% and 0.2% glucose in the encystment medium. No morphological differences were detected in these abortive encysting cells in the presence or absence of cyclic AMP added to the cell culture. Thus the nucleotide apparently did not influence encystment of A. vinelandii when added in high concentrations to the cells. Similar findings with respect to sporulation have been reported by Clark and Bernlohr (20).

The length of time for which the cells were permeable to cyclic AMP is unknown. Presumably, treating A. vinelandii cells with Tris-EDTA affected the membrane

for a period up to 4 hours, as judged by the effect of Tris and Tris-EDTA on nitrogen fixation. No direct tests were made of the intracellular concentrations of cyclic AMP but extracellular concentration of the nucleotide and conditions for increased membrane permeability which are effective in reversing catabolite repression in other gram-negative organisms did not overcome abortive encystment in A. vinelandii.

The replacement of glucose with BHB induces greater than 90% of the cells of A. vinelandii to convert to cysts and thus permits the study of the time course of the biochemical changes which occur during this cellular differentiation process. Two early biochemical events following the initiation of encystment were (1) the rapid decrease in the rate of nitrogen fixation, and (2) the reduction in the activity and ultimate loss of glucose-6-phosphate dehydrogenase. The nitrogen-reducing enzyme complex is membrane-associated in A. vinelandii (23, 53, 74) and requires pools of ATP (36) and reduced nucleotides (6, 115) which are produced by the oxidation of substrates such as glucose, acetate, and propanol. In the absence of any carbon source, low levels of nitrogenase activity were observed which then decreased to zero as the cells' endogenous substrate reserves were exhausted. The cessation of nitrogen fixation of cells in the presence of either BHB or crotonate as substrates therefore constitute

a paradox since either compound can be oxidized by A. vinelandii cells to the equivalent of two acetate molecules and ultimately to CO_2. Furthermore, the enzyme BHB dehydrogenase in the cell induced by BHB is similar to that which is in cells grown in acetate (43, 44, 89) and thus it would be expected that similar yields of ATP and reduced nucleotides would occur. Pretreatment of the washed vegetative cells with Tris-EDTA enabled the cells to reduce acetylene (nitrogen fixation) in the presence of BHB. This indicated that BHB could provide the necessary ATP and reduced nucleotides for nitrogen fixation (6). Therefore, it appears likely that, upon the induction of encystment with BHB, certain properties of the membrane may have been altered so that nitrogenase could no longer function. The putative alteration may have been dependent on achieving low levels of divalent cations in the membrane because nitrogen fixation occurred in the presence of BHB in cells which had been pretreated with Tris-EDTA. These same cells then lost their nitrogen-fixing ability after 4 hours in Burk's buffer from which they presumably regained their complement of divalent cations.

The two cyst coats, the exine and the intine, each contain protein, lipid and carbohydrate (55, 56). The precursors of these coats could have been derived

from either cell constituents present at the time at which encystment was initiated or from BHB, the inducer of encystment. Since radioactive BHB was observed to be incorporated into cells, some or all of the protein and carbohydrate precursors for cyst coats could have been derived from BHB. The synthesis of the coat subunits from BHB, particularly the carbohydrates, would require the functioning of enzymes in the glyoxylate shunt and gluconeogenesis. These include isocitrate dehydrogenase, isocitrate lyase, malate synthase, aldolase and FDPase which were indeed found to be induced in a particular sequence during the course of encystment. The two enzymes of the glyoxylate shunt are also found to be induced during microcyst formation in Myxococcus xanthus (10). The enzymes of the glyoxylate shunt and the TCA cycle are induced during sporulation in the Bacillaceae (37, 38, 64, 70). Taken in total, these findings suggest a strong similarity in metabolic patterns which exists in the differentiation of these procaryotes. In all cases, the cells appear to be in a metabolic shift-down and convert non-carbohydrate substrate(s) into proteins and carbohydrates via the glyoxylate shunt, the TCA cycle and gluconeogenesis.

Time-lapse phase contrast microscopy in conjunction with the determination of the DNA content per cell (89) show that encysting cells undergo a final round of

DNA synthesis before cell division. Using tracer techniques, it was confirmed that cells finished the synthesis of new DNA at about the fourth hour of encystment, which is concomitant with or just precedes cell division. RNA synthesis, however, continued until about the twelfth hour and then plateaued, with no further net synthesis. Using BHB-3-^{14}C, the distribution of radioactivity into the RNA fraction also increased until the twelfth hour and then began to decrease. These results are similar to the events in sporulation where the cessation of DNA synthesis precedes cell division (39) and where RNA synthesis and turnover continues for an extended period. Protein synthesis continued throughout encystment, as shown by the incorporation of ^{14}C-leucine and ^{14}C-BHB into encysting cells, and by the occurrence of peaks of certain enzyme activities which appeared late in the differentiation process. The appearance of new enzymes indicate that new messenger RNA must be made throughout encystment, and hence, turnover of RNA must occur.

In studying the rate of protein synthesis, it was observed that encysting cells of A. vinelandii were more resistant to chloramphenicol than were vegetative cells. At a concentration of 100 µg chloramphenicol per ml, ^{14}C-leucine uptake in vegetative cells was reduced by 90%. Bernlohr and Webster (7) also noted that 10^{-3} M (323 µg per ml) chloramphenicol inhibited ^{14}C-glycine

incorporation into the DNA, RNA and proteins of A. agilis O (A. vinelandii) grown in the presence of sucrose. However, in encysting cells, 100 µg of the antibiotic per ml reduced the rate of protein synthesis by only 25%.

The course of encystment and the formation of PHB granules, as viewed by phase-contrast microscopy, were not altered. By contrast, chloramphenicol inhibits sporulation (88) and only 10 µg of the antibiotic per ml can prevent the synthesis of PHB granules in spores (47). This striking difference between the two differentiation processes may be related to differences in the permeability of the respective cells to the antibiotic.

Encysting cells which were pretreated with Tris or Tris-EDTA were as resistant to chloramphenicol as untreated encysting cells. This supported the earlier observation that EDTA could increase the cells' permeability to unrelated compounds (actinomycin D, o-nitrophenyl galactoside, and carbamyl phosphate) without affecting growth rate or affecting active transport systems in the membranes (as shown by using ^{14}C-amino acid incorporation studies) (52).

Cells encysting in the presence or absence of chloramphenicol were fractionated to determine the extent of ^{14}C-leucine incorpation into proteins and the cell extracts were examined by SDS-gel electrophoresis to determine whether aberrations had occurred in the

leucine distribution into peptides. More than 90% of the ^{14}C-leucine incorporated into encysting cells was in the protein fraction. The results of SDS-gel electrophoresis confirmed that ^{14}C-leucine was incorporated into protein subunits during encystment in the presence or absence of chloramphenicol. The pattern of distribution of the radioactivity into the protein subunits which were separated in the gels was qualitatively the same as the total protein subunits stained with Coomassie brilliant blue. This suggested that ^{14}C-leucine was incorporated into all the proteins rather than into one or a few species during encystment in the presence of the antibiotic. A comparison of the gel scans of protein subunits from cells grown in the presence or absence of chloramphenicol revealed there were no qualitative and little quantitative differences in the various protein subunit bands. This again supported the earlier observation that concentrations of up to 100 μg of the antibiotic per ml did not change the kinds of proteins synthesized nor the course of morphogenesis in encysting cells.

The resistance to chloramphenicol in encysting cells may be due to reduced permeability of the cell membranes to the antibiotic. Chloramphenicol inhibited synthesis of β-galactosidase in osmotic lysates of E. coli cells which were chloramphenicol-resistant as a

result of multistep mutations from a sensitive parent, and in E. coli cells with the resistance factor (R-factor). However, in intact cells of the resistant strains, penicillin spheroplasts and lysozyme protoplasts, net synthesis of protein and β-galactosidase were not inhibited by the antibiotic. Because both the spheroplasts and protoplasts exhibited chloramphenicol resistance, these results point out that the membrane rather than the cell wall serves as the permeability barrier to chloramphenicol (61, 106).

The effect of BHB or its derivatives could be quite direct and due to its incorporation into the lipid fraction, in particular the phospholipids of the encysting cell membrane. The encysting cells were analyzed to see if BHB-3-^{14}C was incorporated into the phospholipids, which in turn could influence certain membrane-associated properties. Within one-half hour after the induction of encystment 45% of the total BHB-3-^{14}C incorporated into the cell was found in the phospholipid fraction. In the same time period, nitrogen fixation had been reduced by 90%. Either BHB or crotonic acid incorporation into the fatty acids of the phospholipids could generate positional or geometric isomers of the normally occurring unsaturated fatty acids of the membrane or could alter the charge distribution of any particular phospholipid being synthesized at a given time. Alternatively, L-BHB could enter the lipid synthetic pathway as L-BHB acyl carrier

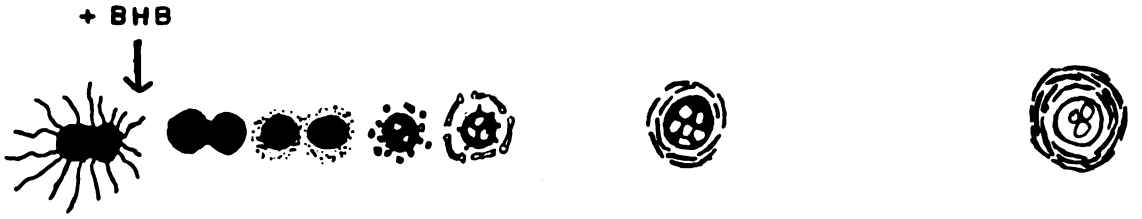
protein. This isomer could not be dehydrated by enoyl acyl carrier protein dehydratase to form crotonyl acyl carrier protein but might participate in further steps of lipid synthesis yielding an L-fatty acid which was hydroxylated at the -1 position. Any of these modifications could then have a profound effect on the stacking of the fatty acid side chains which, in turn, could have an effect on membrane-related properties such as permeability or the activities of certain membrane-associated enzymes.

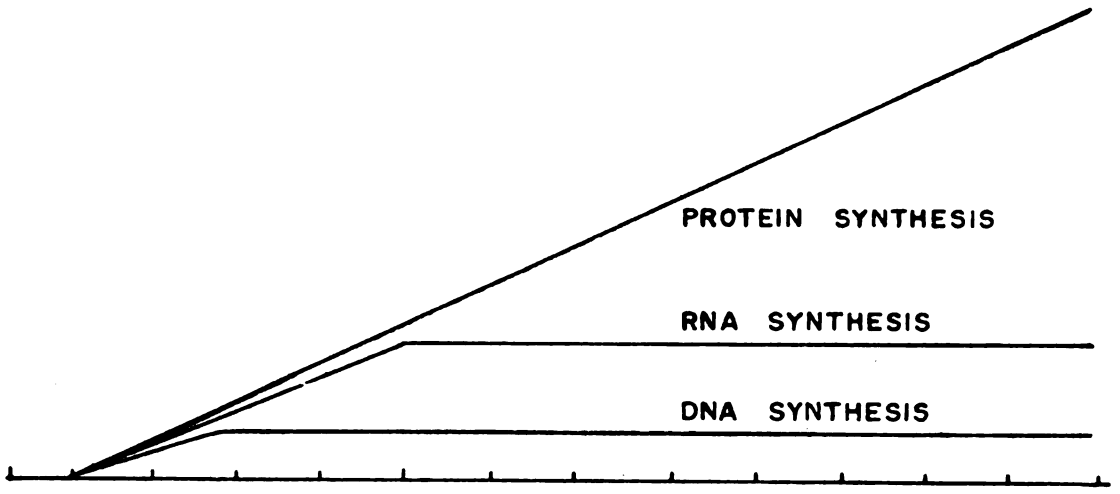
An alternative explanation for the chloramphenicol resistance would require the very rapid induction of a chloramphenicol acetylating enzyme (73) mediated by the addition of BHB or its metabolites. However, this suggestion seems less likely because cells cultivated in the presence of glucose, propanol or acetate were all sensitive to chloramphenicol.

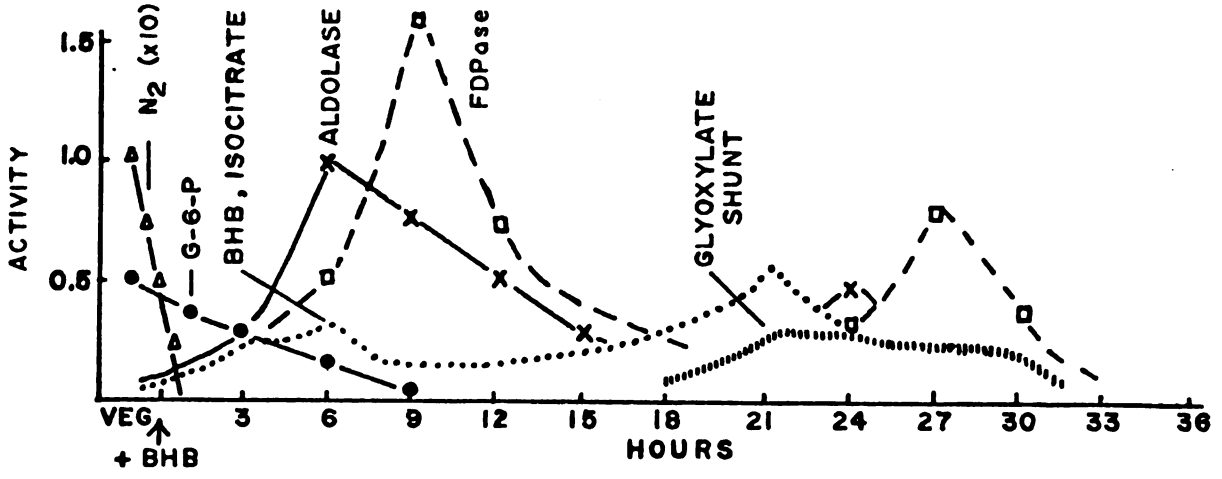
The sequential morphological changes which occur during encystment have been described (40). The biochemical events which take place during this differentiation process may be correlated with the morphological changes, and together the data provide the basis for the division of encystment into several stages. The following schematic diagram (Figure 18) is the compilation of the morphological and biochemical events which occur upon the induction of encystment. Upon the addition of BHB

Figure 18.--A schematic diagram of the morphological and biochemical events during the course of encystment of A. vinelandii.

See text for the description of the diagram.


+ BHB


PROTEIN SYNTHESIS
RNA SYNTHESIS
DNA SYNTHESIS


ACTIVITY
1.5
1.0
0.5
N2 (x10)
G-6-P
BHB, ISOCITRATE
ALDOLASE
FDPase
GLYOXYLATE SHUNT
VEG
+ BHB
3
6
9
12
15
18
21
24
27
30
33
36
HOURS

to a culture of A. vinelandii, several biochemical changes are immediately apparent: (1) a rapid decrease of activities of enzymes associated with vegetative growth (nitrogenase and glucose-6-phosphate dehydrogenase) and a rapid increase of new enzyme activities (BHB dehydrogenase, isocitrate dehydrogenase and the glyoxylate shunt enzymes). Furthermore, cells display a greater resistance to chloramphenicol than vegetative cells. (2) At 4-6 hours of encystment, cells undergo a final round of cell division and form precysts which appear round and non-flagellated (non-motile). DNA synthesis ceases and the DNA content per cell remains constant from this time until the completion of encystment. (3) The activities of the two gluconeogenesis enzymes (aldolase and FDP_{ase}) reach peak values at 6 and 9 hours, respectively. (4) By the twelfth hour, net RNA synthesis ceases. Electron micrographs show that also at this time, several developing exine layers are observed along the inner edge of the capsular material. (5) Peak activities of BHB dehydrogenase, isocitrate dehydrogenase, isocitrate lyase, malate synthase reappear at 21 hours, followed by aldolase at 24 and FDP_{ase} at 27 hours. Protein, lipid, and PHB syntheses continue throughout encystment. (6) By 36 hours, the cells are metabolically dormant and are endowed with the ability to withstand adverse environmental conditions. Electron micrographs reveal that at

this time the cells are mature cysts whose central bodies are encased by multilayered exine coats along the outer edges of the capsular matrix.

In the absence of nitrogen fixation macromolecular synthesis during encystment of A. vinelandii must depend on the turnover of both RNA and protein. In this sense encystment appears like sporulation except that encystment occurs over a much broader time span. A further fundamental similarity in the two differentiation processes is the early completion and cessation of DNA synthesis followed by the segregation of sister nucleotides into separate cells (39). This leads to the formation of one spore in Bacillaceae but two cysts in Azotobacteriaceae. Both developmental processes are also characterized by the appearance of new enzyme activities and the synthesis of new components characteristic of resting cells. The increase in activities of enzymes of the TCA and glyoxylate shunt are common during early stages of sporulation in Bacillus and during encystment in Azotobacter and Myxococcus. The parallel which is seen in these biochemical events and the time course for their appearance suggests that there may exist a general pattern of control of differentiation in the procaryotes.

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