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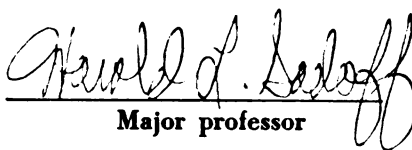
ENOLASE FROM BACILLUS LICHENIFORMIS
PURIFICATION, PROPERTIES AND REGULATION
OF THE ENZYME

presented by

Mark Edward Ruppen

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ENOLASE FROM BACILLUS LICHENIFORMIS.
PURIFICATION, PROPERTIES AND REGULATION
OF THE ENZYME

By

MARK EDWARD RUPPEN

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ABSTRACT

ENOLASE FROM BACILLUS LICHENIFORMIS. PURIFICATION, PROPERTIES AND REGULATION OF THE ENZYME

by

MARK EDWARD RUPPEN

Enolase has been purified from Bacillus licheniformis. The enzyme preparation was homogenous as judged by polyacrylamide gel electrophoresis of native and denatured protein. The molecular weight of the enzyme is 370,000 daltons and it is an octamer composed of 8 identical subunits with a molecular weight of 47,000 daltons. The enzyme is very thermostable in the presence of Mg^{+2} . The pH optimum is 8.0 in Tris buffer. The K_m for 2-phosphoglyceric acid is 0.4 mM.

An increase in the rate of protein turnover could be demonstrated after the cessation of growth in a minimal medium. Enolase activity remained constant throughout early sporulation and the amount of enolase present in these cells was the result of a balance of synthesis and degradation of the enzyme.

In a supplemented nutrient broth medium enolase activity reached a maximum value at T_2 and then

decreased during sporulation. The rate of decline of enolase activity parallels the rate of the loss of immunologically cross reactive enolase protein. This rate of loss is the same as the general turnover rate and can be blocked by the early addition of chloramphenicol or 2,4-dinitrophenol. This suggests that the enolase degradation system is induced during sporulation and requires energy and indicates a link between enolase degradation and general protein turnover.

Enolase synthesis could be detected throughout sporulation. The highest rates of synthesis were found during exponential growth and the early stages of sporulation. Subsequently, the rate declines until; at T_8 , the rate is only 10% of the maximum value.

Several models are presented that may explain the regulation of the amount of enolase in the developing spore.

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INTRODUCTION

Endospore formation in the genus Bacillus is a well studied model of microbial differentiation. During the course of sporulation, selective gene transcription is expressed resulting in dramatic biochemical and morphological changes leading to the formation of a dormant spore. Several excellent reviews have been written describing the biochemical and morphological events of sporulation (14,29).

One of the more interesting biochemical changes that occur during sporulation is a dramatic shift in the cell's metabolism. At the initiation of sporulation, the metabolic pattern shifts from glycolysis and amino acid biosynthesis to gluconeogenesis and amino acid catabolism (38). Coincident with the onset of amino acid catabolism is an increase in the rate of protein turnover. The rate of protein turnover increases during sporulation from a rate less than $3\% \text{ hr}^{-1}$ to $8-20\% \text{ hr}^{-1}$ (13). Proteins synthesized during sporulation are assembled from turnover of vegetative cell protein.

Early studies suggested that there was no specificity to protein turnover (56). Subsequent studies have demonstrated that there is some degree of specificity.

Work by Switzer's group has demonstrated that enzymes involved in nucleotide biosynthesis are selectively inactivated and degraded early in the sporulation process (60). Work by Deutscher and Kornberg (12) has shown that the profiles of various vegetative cell enzyme activities are radically different during sporulation. Therefore, there is some degree of specificity in protein turnover.

It is known that enzyme activities found in the spore are also present in the vegetative cell (12). In at least one case, it has been shown that the spore enzyme and the vegetative enzyme are the products of the same gene (17). Since cells and spores share many of the same enzyme activities, it would be interesting to investigate the possibility of specific protein turnover during sporulation. There are at least two possible mechanisms of attaining the proper enzyme complement in the spores. The "shared" enzymes could be synthesized and degraded continuously during sporulation and, through some unknown mechanism, are compartmentalized into the spore. This mechanism would require no specificity to protein turnover with regards to the ultimate protein composition of the spore. On the basis of biological economy, it would seem that a cell, especially a starving sporulating cell, has

little to gain by simultaneously degrading and synthesizing a particular protein.

The second, and more economic approach, would be for the sporulating cell to spare the vegetative proteins from proteolytic degradation and sequester it, intact, into the spore. This type of mechanism would require that some degree of specificity would exist in protein turnover.

This thesis presents the results of a study of the enzyme enolase (phosphopyruvate hydratase. E.C. 4.2.1.11) in Bacillus licheniformis. Enolase was chosen for this study because it is required during growth, sporulation, and germination, and studies of the B. megaterium enzyme suggested that milligram quantities of pure enzyme could be easily obtained (52). Pure enzyme was required to produce the necessary specific antibodies that were used to precipitate enolase from a crude extract, thereby allowing a detailed study of the extent of enolase synthesis and degradation during sporulation.

The type of investigation conducted was planned to yield an insight into the mechanism of protein turnover and compartmentalization during sporulation. One of the specific questions that was dealt with was: Does enolase activity represent a balance between synthesis and degradation, or is the enzyme stable and not subject

to protein turnover during sporulation? The results indicate that enolase (and possibly other enzymes destined for inclusion in the mature spore) is not immune to degradation and that the amount of enzyme protein present during sporulation represents a balance between the synthesis and turnover of enolase.

LITERATURE REVIEW

This literature review deals primarily with protein turnover and enzyme inactivation during sporulation. I will attempt to discuss those facets of these topics which are most relevant to my study. Excellent reviews are available on each of these topics (13,59). In order to familiarize the reader with the subject of sporulation, a short description of the process will be included in this section.

Sporulation in the Genus Bacillus

Sporulation is initiated at the end of exponential growth and is due to the exhaustion of certain nutrients from the growth medium (46). Catabolite repression of various functions is relieved but the release of catabolite repression alone is not sufficient to initiate sporulation. Hence, the identification of sporulation specific functions is difficult (40).

There are many morphological and biochemical changes associated with sporulation. The differentiation process is sequential and has been, on the basis of morphology, divided into 7 stages (14). The initial stage, stage 0, is the end of exponential growth and is designated as T_0 . Each hour subsequent to T_0 is designated T_1 , T_2 , etc.

The time period from T_0 through T_2 covers the final expression of purely vegetative functions and is when the initiation of sporulation occurs. During stage II (T_2), a final modified cell division occurs producing an asymmetric septum to yield the prespore (the smaller daughter cell) (25). During stage III (T_3 - T_4), engulfment of the prespore region by an "inside out" membrane forms the forespore which is now isolated from the mother cell compartment. During stages IV, V, and VI, the cortex and spore coat are deposited, and increases in the refractility and heat resistance are noted. During stage VII (T_8 - T_{10}), lysis of the mother cell occurs and the mature spore is released (14).

Protein Turnover During Sporulation

Protein turnover occurs in all members of the genus Bacillus studied with the possible exception of B. brevis (54). While the role of turnover during the sporulation process has not been clearly elucidated, much of the biochemistry and physiology of protein turnover has been defined.

At the end of exponential growth, the rate of protein turnover increases from less than 3% per hour to as much as 20% per hour. The rate varies with the type of medium and the species of Bacillus. The highest rates of turnover have been measured in B. licheniformis and

B. subtilis, 20% and 18% per hour, respectively (13). However, during sporulation in B. brevis, the rate of turnover is very low (1.6% per hour) or absent.

The following reasons emphasize that the above values for turnover rates represent the minimal rates of turnover. The method most commonly used to measure turnover rates is the classic "pulse-chase" experiment (56). In this kind of experiment, radioactive amino acids are used to label the cell proteins. After a certain period of time, a large excess of unlabeled amino acid is added to the culture, thereby effectively diluting the labeled amino acid. Theoretically, this "chase" should stop the incorporation of labeled amino acid into the cell's protein. However, it has been shown that sporulating cells do not exchange endogenous amino acids with exogenously added amino acids (4). Hence, the re-utilization of amino acids generated by proteolysis cannot be completely excluded and, therefore, this kind of experiment yields erroneously low turnover rates. By the use of an H_2^{18}O exchange method that allowed measurements of turnover in excess of 100%, and is not subject to permeability restrictions, Bernlohr concluded that B. licheniformis turns over 200% of its protein during sporulation (5).

The role of protein turnover in sporulation may be

very complex. The following possibilities have been considered: 1) Protein turnover (as general proteolysis) might supply the sporulating cell with endogenous amino acids during a time of starvation. These cells actively carry out amino acid catabolism and examination of respiration rates have suggested that they derive a great deal of energy from oxidizing the amino acid carbon skeletons (3). Sporulating cells also synthesize protein and free amino acids are needed. Since some amino acid biosynthetic enzymes disappear during sporulation (and therefore the capability of de novo amino acid synthesis) amino acids must be derived from proteolysis if protein synthesis is to occur (21). 2) Protein turnover (selective or limited proteolysis) may cleave various repressors allowing expression of sporulating genes (40). Selective limited proteolysis is also involved in the post-translational processing of proteins. Cleavage of a spore coat protein precursor has been well documented in B. cereus and in B. subtilis (8,37). The enzyme involved in the cleavage is the major intracellular serine protease. Also in B. cereus, the vegetative form of aldolase is acted on by a protease to yield the sporulation form of aldolase (45). The mechanism of this modification has not been further studied.

Not all proteins are degraded at the same rate during sporulation. Thus, the average turnover rate is a reflection of the rapid turnover of some proteins and the relatively slow turnover of other proteins. The information on turnover rates of individual proteins is limited. Maurizi and Switzer have found that the half-life of aspartic transcarbamylase (ATCase) is approximately 1.5 hours as compared to a half-life of 6 hours for the average protein calculated by using the average turnover rate (35).

Enzyme activity profiles during sporulation have also demonstrated the heterogeneity of turnover rates (assuming that enzyme activity corresponds to the amount of enzyme protein present). Some enzyme activities rapidly disappear during sporulation while other activities slowly decline from the levels present in exponentially growing cells. The activities of ATCase, glutamine-PRPP amidotransferase (62) and carbamyl phosphate synthetase (39) disappear during sporulation while the activity of arginase, ornithine transcarbamylase (12) extracellular protease (2) and dipicolinate synthetase (26) increase during sporulation. The activities of DNA polymerase and adenylate kinase decrease to a lower value than that observed in exponentially growing cells (12).

Using pulse-chase techniques, it can be calculated that approximately 10% of the protein in the completed spore is derived intact from the vegetative cell (29). The fact that the forespore septum engulfs approximately 20% of the volume of the cytoplasm tends to suggest that protein engulfed by the spore septum may be spared from proteolysis and conserved in the spore. Several workers have demonstrated that protein turnover is negligible in the developing spore (15). Pulse-chase experiments have shown that the amount of radioactivity incorporated into the spore proteins is constant at a time when a turnover rate of 18% per hour can be demonstrated in the mother cell compartment. The hypothesis, although simple, has pitfalls and several important points must be taken into consideration. The efficiency of the chase of unlabeled amino acid is unknown. The incorporation of label into spore protein suggests that labeled amino acids can enter the forespore but this does not preclude the possibility that amino acids are unable to leave the forespore. If this were the situation, the efficiency of a chase would be limited and turnover could not be demonstrated. Since proteolytic enzymes have been found in the developing spore (47) exclusion of proteases cannot be a mechanism for the conservation of the engulfed protein.

Until the question of amino acid permeability can be answered, "compartmentalized turnover" must be greeted with some skepticism.

The enzymes involved in protein turnover are thought to be the intracellular serine proteases (13). There is an amount of literature concerned with the characterization and roles of the proteases from the various bacilli. As more work is published it is becoming evident that the role of these proteases is not quite as clearly defined as it was once thought to be. An excellent review on proteases has been written by Doi (13).

In B. licheniformis, there appears to be a major intracellular serine protease similar to the intracellular protease of B. subtilis (57). Synthesis of the protease is regulated by both carbon and nitrogen catabolite repression (6). Since B. licheniformis lacks a well developed genetic system, little work has been carried out with protease mutants. Therefore, studies with B. subtilis and B. cereus protease mutants will be discussed. The number of true protease mutants are few due to the occurrence of pleiotropic mutations that are common in sporulation mutants. The studies presented below represented the best biochemical studies of the roles of the intracellular proteases in the sporulation

process.

Hageman and Carlton isolated two mutants of B. subtilis with greatly reduced amounts of protease activity (22). These mutants were asporogenic. An interesting observation is that in one of the mutants, the inactivation and degradation of ATCase proceeded normally (36). This observation suggests the possibility of a secondary degradation system that may function in lieu of the major intracellular protease. Cheng and Aronson isolated a mutant of B. cereus lacking intracellular protease activity (9). A small amount of activity related to a minor protease was still present in this mutant. This mutant was asporogenic, exhibited a reduced rate of protein turnover, and failed to cleave a precursor of the spore coat protein.

There are other enzymes in the sporulating cell that have the ability to cleave peptide bonds (47). Various esterases, peptidase, and amidases have been identified. Very little work has been done on the role of these enzymes in sporulation and protein turnover.

Enzyme Inactivation During Sporulation

At the onset of sporulation, the activity of several enzymes begins to decrease at a rate greater than the rate of general protein turnover (12). It appears that these enzymes are subject to specific rapid

inactivation processes.

Work by Switzer's group has been directed towards analyzing the mechanisms and controls of some enzyme inactivations (35,36,62,63). Their work with the enzyme aspartic transcarbamylase (ATCase) represents the most complete study of enzyme inactivation during sporulation.

Upon the cessation of exponential growth, ATCase activity disappears with a half-life of approximately 2 hours (36). The inactivation can be blocked by inhibiting energy metabolism with 2,4-dinitrophenol but cannot be blocked by inhibiting RNA or protein synthesis with the addition of rifampin or chloramphenicol at T_0 (36). The progress of the inactivation is normal in a series of mutants blocked at stage 0 of sporulation (spo 0 mutants) and in protease deficient mutants (35,36). Immunologically cross reacting, enzymatically inactive protein fragments could not be detected in the course of a thorough immunological search (36). Although the mechanism of the enzyme inactivation process is unknown, inactivation always occurs at the cessation of growth, suggesting that a growth related signal may be involved in the inactivation. Since ATCase degradation is normal in protease-less mutants, the possibility of a proteolytic system with restricted specificity or the involvement of other peptide bond cleaving enzymes must be considered

as means of degradation.

The glutamine-PRPP amidotransferase of B. subtilis is also inactivated at the beginning of sporulation but this process has not been as well characterized as the ATCase system. The inactivation appears to involve the oxidation of an Fe-S cofactor (62).

Inactivation of ATCase and amidotransferase effectively blocks pyrimidine and purine synthesis during sporulation. The lack of nucleic acid biosynthesis may be a contributing factor to the dormancy of the spore (48). The inactivation and degradation during sporulation of specific enzymes at rates different from general protein turnover rates exists. This level of specificity is unknown but could be related to the "native or denatured" conformation of proteins, (i.e., a denatured protein is more susceptible to proteolysis) or it could function at the level of the protease (i.e., a protease with restricted specificity such as the rec A gene product (6,43)).

MATERIALS AND METHODS

All common reagents were of analytical grade quality and are routinely available. The following reagents and materials were obtained from the following sources: D-2-phosphoglyceric acid, Na⁺ salt, antipyrine, diacetylmonoxime, phenylmethanesulfonyl-fluoride, N-carbamyl-DL-aspartic acid, m-aminophenyl-boronic acid, carbamyl phosphate, Li⁺ salt (Sigma Chemical Co.); [4,5]-³H-leucine (Schwarz-Mann); U-¹⁴C-leucine (New England Nuclear); Freund's adjuvant and nutrient broth (Difco).

Rabbits were obtained through and maintained by the Michigan State University Laboratory Animal Care Services. Six month old New Zealand white rabbits (both male and female) were used in this study.

Bacterial Strains and Media

Experiments in this study were conducted using B. licheniformis A-5 originally obtained from R. W. Bernlohr. Cells were grown in the glucose-ammonia-salts medium (A medium) of Siegel, Donohue and Bernlohr (51). Supplemented nutrient broth medium (SNB) consisted of A medium to which 8g/l of nutrient broth had been added.

The glucose (3M, 200X) ammonia ($2\text{M}(\text{NH}_4)_2\text{SO}_4$, 200X), and the salts solution (500x) were autoclaved separately. Massive precipitation could be avoided if the salt solution was added aseptically to the sterile buffer - nutrient broth mixture at room temperature.

Cultivation of the organism was carried out as follows. 25 ml of SNB were inoculated with about 10^7 spores and incubated overnight with shaking at 37°C . This culture was used as an inoculum. A 1% volume of inoculum was transferred into fresh prewarmed media and incubated with shaking at 37°C . Growth was monitored by measuring turbidity using a Klett-Summerson colorimeter with a number 66 filter.

Harvesting and Preparation of Cell Free Extracts

25 ml samples were withdrawn from a one liter culture during growth and sporulation and were immediately centrifuged at $12,000 \times g$ for 5 minutes at 4°C . Cell pellets were washed with ice cold 1 M KCl and recentrifuged. This washing procedure effectively removed any surface-associated proteases that were secreted during sporulation. The washed pellet was then frozen and stored at -20°C .

For preparation of cell free extracts, the pellets were first suspended in 1 ml of 0.05 M tris - 0.005 M MgCl_2 - 0.01 M 2-mercaptoethanol - 0.001 M

phenylmethylsulfonylfluoride (PMSF), pH 7.6. The cells were then broken by sonication in an MSE Sonicator. Four 30-second pulses with 30 second cooling periods were sufficient to disrupt greater than 90% of the cells. Samples were cooled in an ice water bath throughout the entire procedure. Cell debris was removed by centrifugation at 48,000 x g for 20 minutes. The supernatant fluid was decanted and immediately assayed.

Enolase Assay

The assay of Singh and Setlow (52) was used to measure enolase activity; however, the 2-PGA concentration was increased to 4.4 mM. The basis of this assay is the increase in the absorbance at 240 nm caused by the formation of phosphoenolpyruvate (PEP). One unit of activity is defined as 1 μ mole of PEP formed / min at 30°C. In this assay, PEP has an extinction coefficient of 13,700 cm^2/M .

Aspartic Transcarbamylase Assay

Aspartic transcarbamylase was assayed using the procedure of Waindle and Switzer (63). Carbamyl aspartate was determined by the method of Prescott and Jones (41).

Extracellular Protease Assay

Proteolytic activity was determined in cell free culture supernatant fluid with Azocoll as the substrate. The assay mixture contained in a 1 ml volume: 0.1 M Tris - 0.01 M CaCl_2 - 10 mg/ml Azocoll. 20 microliters of supernatant fluid was added to initiate the reaction. After 10 minutes incubation at 30°C with shaking, the assay was terminated by placing the tubes on ice. The insoluble substrate was removed by centrifugation. The absorbance of the supernatant was determined at 520 nm. 1 unit of activity is equal to a change in absorbance of 1.0/10 minutes.

Protein Determination

The protein assay of Kalb and Bernlohr (27) was used throughout this study to estimate the protein concentration in crude extracts and in purified enolase preparations. Protein solutions were diluted in distilled water.

For the determination of whole cell protein, 20 ml of culture were withdrawn and 2.2 ml of 100% w/v trichloroacetic acid (TCA) were added. After 20 minutes on ice, the sample was centrifuged at 12,00 x g and the supernatant fluid was discarded. The pellet was washed with 10 ml of cold 10% TCA, recentrifuged, and the supernatant fluid was discarded as before. Two

milliliters of a 0.2 N NaOH were added to the pellet and the suspension was heated for 1 hour at 80°C. Insoluble debris was removed by centrifugation and the resulting supernatant was assayed for protein by the method of Lowry et al. (32), using bovine serum albumin as a standard.

Polyacrylamide Gel Electrophoresis

Electrophoresis of native protein was carried out using the method of Davis (11). Sodium dodecyl sulfate (SDS) gel electrophoresis was performed using the method of Weber and Osborn (64) or of Laemmli (30). Protein bands in the gels were visualized using 0.02% Coomassie blue R-250 in water-ethanol-acetic acid (44).

Immunochemical Methods

Administration of the antigen. Purified enolase (1 mg/ml) in buffer was added to an equal volume of Freund's complete adjuvant. The mixture was sonicated for several minutes using a microprobe until a stable emulsion was obtained. The emulsion was administered intramuscularly into a rabbit's hindquarters. Two weeks after the first injection, the rabbit received a booster shot (1 mg pure enolase in buffer) subcutaneously in the neck region.

The rabbits were bled via the marginal ear vein. After shaving the ear, the area was washed with ethanol

and xylene was applied as a vasodilator. The vein was nicked longitudinally with a sterile lancet and the blood collected in the centrifuge tube. Forty to fifty milliliters of blood could be collected at 1 week intervals.

The whole blood was allowed to stand at room temperature in the centrifuge tube into which a wooden applicator stick had been inserted. After the blood had clotted, the tube was placed overnight in the refrigerator. The clot, which had collapsed around the applicator stick, was withdrawn. The serum was clarified by centrifugation at 12,000 x g for 10 minutes and was stored at -20°C.

Purification of immunoglobulin G (IgG). One volume of a saturated ammonium sulfate solution was added to the clarified serum with stirring. After 20 minutes at room temperature, the precipitated protein was collected by centrifugation and suspended in one volume of 0.01 M Na-phosphate buffer, pH 7.0. The precipitation with ammonium sulfate was repeated and the pellet dissolved in the phosphate buffer and then dialyzed overnight versus 20 volumes of buffer. The protein was applied to a 1.5 x 30 cm diethylaminoethyl (DEAE) cellulose column equilibrated with the phosphate buffer. IgG does not bind to the column

under these conditions and elutes in the wash volume. Protein in the wash was precipitated by the addition of 1 volume of saturated ammonium sulfate. The protein was finally dissolved in and dialyzed against 0.05 M Tris - 0.005 M MgCl_2 , pH 7.6 and stored at -20°C .

Quantitative precipitation. Determination of the equivalence point was performed using a quantitative precipitation test in which the amount of antibody was held constant and the amount of enolase was varied (10). Antibody capable of precipitating approximately 1 unit of enolase was added to a 400 μl microfuge tube (Beckman). Increasing amounts of enolase, either pure enzyme or a crude extract, and buffer (0.05 M Tris - 0.005 M MgCl_2 , pH 7.6) were added to give a final volume of 0.20 ml. After incubation overnight at 4°C , the tubes were spun and the supernatant fluid was assayed for enolase activity. The equivalence point is the tube where enolase activity is first detected in the supernatant fluid.

Antibody neutralization. This test can be used to quantitate the amount of antigen present in a sample and detect the presence of enzymatically inactive cross reacting material (24). This method is essentially the reverse of the quantitative precipitation method described above. The amount of

antibody was varied while the amount of enolase remained constant. Usually, crude extracts containing 1 unit of enolase activity, varying amounts of antibody and buffer, were added to a microfuge tube maintaining a constant volume of 0.2 ml. After overnight incubation at 4°C, the samples were spun and enolase activity remaining in the supernatant fluid was determined. The amount of antibody was such that a linear loss of enolase activity versus increasing amounts of antibody could be plotted and the slope of the loss was determined graphically.

Ouchterlony double diffusion tests. Double diffusion tests were performed on microscope slides coated with approximately 4 ml of 1% Ionagar in 0.05 M Tris - 0.005 M MgCl₂ pH 7.6. Wells were punched into the hardened agar and the antigen and antibody added. The slides were incubated in a moist atmosphere at room temperature overnight. The slides were then washed for several hours in 0.85% NaCl, followed by distilled H₂O, and finally stained with Coomassie blue for increased visualization of the precipitin lines.

Immuno-electrophoresis. Immuno-electrophoresis was performed by the method of Campbell (7). A slide was coated with 0.85% Ionagar in 10 mM NaPO₄ buffer,

pH 7.0, and a circular well was cut from the center of the agar and filled with antigen to which bromphenol blue had been added to serve as a marker. Electrophoresis was conducted for 75 minutes at 1.5 mamp per slide. Following electrophoresis, a trough (4 x 50 mm) was cut into the agar and filled with anti-serum. Immunodiffusion was allowed to proceed overnight in a moist atmosphere. The slides were washed, dried, and stained as described above.

Molecular Weight Determinations

The molecular weight of the native enolase molecule was determined using the sedimentation velocity method of Martin and Ames (33). A 5.4 ml linear gradient of 5% to 20% sucrose in 0.05 M Tris - 0.005 M $MgCl_2$, pH 7.6, was prepared in 1 x 3 cm cellulose nitrate ultracentrifuge tubes (Beckman). One hundred-fifty microliters of a solution containing approximately 2 units of enolase, 5 μg of catalase and 10 μg of jack bean urease was layered onto the gradient and the tubes were spun at 35,000 rpm in an SW 50.1 rotor for 6 hours at 4°C using a Beckman L-2 ultracentrifuge. Two drop fractions were collected by puncturing the bottom of the tubes with a hypodermic needle and these were assayed for enolase, catalase, and urease. Catalase activity was measured by

following the decrease in the absorbance at 240 nm due to H_2O_2 reduction (33). Urease was assayed by measuring ammonia with Nessler's reagent. The urease assay mixture contained: 0.8 ml 0.05 M K^+ phosphate pH 7.6 and 0.1 ml 0.05 M urea. The reaction was run for 30 minutes at 30°C and was terminated by the addition of 1 ml of Nessler's reagent. The absorbance at 420 nm was then determined. The molecular weight of catalase was assumed to be 250,000, while the molecular weights of the urease species were 360,000 and 180,000.

Subunit molecular weight was determined using the SDS gel electrophoresis system of Laemmli (30). Protein samples (10-20 μg) were mixed with 1 volume of 0.05 M Tris - 10% 2-mercaptoethanol - 1% SDS and heated at 95°-100°C for 20 minutes. The molecular weight standards used were: ovalbumin (43,000), bovine serum albumin (68,000), and phosphorylase a (90,000).

Growth and *In Vivo* Labeling of Enolase for Immunoprecipitation Studies of the Synthesis of Enolase

A 1 liter culture in a 3 liter Fernbach flask was prepared as previously described. At various times, 25 ml samples were removed and placed into a 250 ml flask. (The transfer was performed as quickly as possible to avoid

perturbing the cells.) Twenty five microcuries of [4-5]-³H-Leucine was added to the flask and incubation was continued. After 20 minutes, unlabeled leucine was added to a final concentration of 4 mM and a 1 ml sample of the culture was removed and pipetted into 4 ml of cold 6.7% TCA. The remaining culture was then quickly chilled in ice water, harvested by centrifugation, washed with cold 1 M KCl, and the cell pellet stored at -20°C. The 1 ml culture sample in TCA was stored at 4°C until needed.

Cell free extracts were prepared as previously described and each sample assayed for enolase activity before immunoprecipitation of the enzyme which was performed using a 3-fold excess of purified IgG. Known amounts (usually 0.5 and 1.0 units) of enolase in the extracts were precipitated in microfuge tubes. An amount of control serum equal to the amount of protein in the anti-enolase serum was added to duplicate tubes with enolase to serve as a control for nonspecific precipitation. After incubation overnight at 4°C, the precipitates were collected by centrifugation for 2 minutes in a Beckman microfuge. The immunoprecipitate was washed three times with 150 mM NaCl and finally dissolved in 50 ml of NCS Tissue solubilizer (Amersham). After incubation overnight at 37°C, the tips of the

tubes containing the dissolved precipitate were frozen in a dry ice-acetone bath and cut off with an electrician's insulation stripper. The tips were placed in scintillation vials and 10 ml of scintillation fluid (ACS, Amersham) was added. After vigorous agitation, the vials were placed in the scintillation counter overnight to allow the chemiluminescence to subside. For the counting of ^3H , the gain was set at 60%, with a window setting of 50-1000. For counting ^{14}C , the gain was 15% and the window setting 50-1000.

The sample of culture that had been taken into TCA was processed in a similar manner. Insoluble material was collected by centrifugation, washed three times with cold 5% TCA, dissolved in NCS Tissue solubilizer and counted.

Labeling of the Cells for Protein Turnover Studies

The most common method of demonstrating protein turnover is to use the "pulse-chase" method of Spudich and Kornberg (56). This technique involves growing the cells in the presence of a labeled amino acid for a certain amount of time followed by the addition of a large excess of unlabeled amino acid. Turnover is measured by the loss of label from the TCA insoluble protein fraction.

Several different techniques were used to label the cells in an attempt to demonstrate protein turnover. In order to measure turnover in cells growing in A medium, the cells were pulsed with U-¹⁴C-leucine (0.1 μ Ci/ml) at a culture density of 10 Klett units (KU). At 40 KU unlabeled leucine was added to a final concentration of 5 mM. Every half-hour, 1 ml samples were withdrawn from the culture and placed into 4 ml of 6.7% TCA. After at least 20 min on ice, the samples were filtered through Millipore GFC filters and washed with 10 ml of 5% TCA - 5 mM leucine and finally with 5 ml of 95% ethanol. The filters were dried (70-80°C oven) and placed in scintillation vials containing 10 ml of ACS scintillation fluid (Amersham). The amount of radioactivity remaining in the insoluble material was measured in a Packard Tri-Carb scintillation counter with the gain set at 15% and the window setting at 50-1000.

The procedure used for demonstrating turnover in cells growing in SNB medium was essentially the same as described above. However, because of variations in the data, several modifications of the labeling procedure were tried in order to obtain consistent results. The following labeling conditions were tried in order to demonstrate turnover. (1) Cells were

labeled in minimal media with U- ^{14}C -leucine (0.1 $\mu\text{Ci/ml}$) and then used to inoculate SNB containing 5 mM leucine. (2) Cells were labeled in SNB media with ^{14}C -leucine followed by the chase (5 mM leucine) in the same flask. (3) Cells were labeled in SNB media with U- ^{14}C -phenylalanine (0.1 $\mu\text{Ci/ml}$) followed by a chase of 5 mM phenylalanine in the same flask. (4) Cells were labeled with either ^{14}C -leucine or ^{14}C -phenylalanine during growth in SNB. After rapidly harvesting and washing with warm SNB, these cells were used to inoculate prewarmed SNB containing a 5 mM chase of amino acid. Of all of these procedures, only when (4) was used to label the cells could turnover be consistently demonstrated. Sampling, filtering and counting were identical to those procedures described for the minimal media.

Determination of Enolase Degradation in Sporulating Cells

The following procedures were employed in an attempt to measure the relative rate of enolase turnover in sporulating cells in A media.

(1) Two parallel cultures were grown in A medium in sidearm flasks. One culture (50 ml) was pulsed with U- ^{14}C -leucine (0.1 $\mu\text{Ci/ml}$) at T_{-1} . The other culture (250 ml) was pulsed with [4,5]- ^3H -leucine

(1.0 $\mu\text{Ci/ml}$) at T_{-1} . At T_0 , unlabeled leucine was added to both cultures to a final concentration of 4 mM. The ^{14}C labeled culture was immediately harvested, washed and stored as a frozen pellet. A 40 ml sample of the ^3H labeled culture was similarly treated. Every hour another 40 ml sample was withdrawn from the ^3H labeled culture, harvested, washed and frozen.

Extracts were prepared from each sample and were assayed for enolase activity. One hundred microliters of the ^{14}C labeled extract was mixed with 400 μl of the ^3H -labeled extract in 1.5 ml microfuge tubes. Fifty microliters of the mixture was removed and mixed with 200 μl of 6.7% TCA. This sample represents the total radioactivity in the total protein fraction of each extract mixture. A volume of antibody capable of precipitating all of the enolase activity was added to the remaining extract mixture. Control tubes containing the same mixture of extracts but treated with control serum were included in each set of experiments. The mixture was incubated overnight at 4°C . The next day both the TCA precipitates and the immunoprecipitates were collected by centrifugation in a Beckman microfuge, washed 3 times with either cold 5% TCA or 150 mM NaCl and finally dissolved in 50 μl

of NCS tissue solubilizer (Amersham) at 37°C. The tips of the microfuge tubes containing the dissolved precipitates were frozen in a dry ice-acetone bath and removed with electrician's insulation strippers. The tips were placed in scintillation vials containing 10 ml of ACS scintillation fluid. The vials were placed in the dark and cold overnight to allow the chemiluminescence caused by the NCS to subside. The radioactivity in each vial was then determined using the following settings on a Packard Tri-Carb scintillation counter: ^3H , gain at 60%, window settings 50-350, 21% spillover of ^{14}C . ^{14}C , gain at 15%, window settings 250-1000, spillover from the ^3H was negligible.

The evaluation of protein turnover by the technique described above relies on the change in the rate of isotopes in samples of total protein and enolase. The ^{14}C -labeled culture serves as an internal standard. In the ^3H -labeled culture, the ratio of label in enolase to that in total protein will reflect the changes in the amount of enolase present in the sample. For a review of this procedure, the report of Quinto et al. is recommended (42).

(2) In this method, the specific radioactivity of enolase was determined. A 250 ml culture growing in A medium was pulsed with [4,5]- ^3H -leucine (1.0 $\mu\text{Ci/ml}$)

at T_{-2} . At T_{-1} a chase (4 mM unlabeled leucine) was added and a 40 ml sample was withdrawn, harvested, washed and frozen. The sampling was repeated at hourly intervals.

Extracts were prepared and enolase activity in each of the extracts were determined. A known amount of enolase (2 to 3 units) was quantitatively precipitated with a 3-fold excess of anti-enolase serum. After overnight incubation at 4°C the precipitates were collected by centrifugation, washed and counted as described above.

RESULTS

Purification of Enolase

Enolase was purified from B. licheniformis using several modifications of the procedure of Singh and Setlow (52). All buffers and ion exchange resins were adjusted to the indicated pH's at 4°C. All enzyme purification steps were carried out at 4°C.

One hundred liters of SNB medium was prepared and inoculated with 3 liters of exponentially growing cells in SNB. The cells were grown in a 100 liter fermentor (Stainless and Steel Co.) at 37°C with vigorous aeration (100 liter of air per minute). The culture was harvested using a Sharples continuous flow centrifuge beginning at T_1 and was completed at T_2 . The cell paste (about 400 gm) was washed once with cold 1 M KCl-1 mM PMSF and frozen at -20°C.

For enolase purification the cells were first suspended in 1 volume of 0.05 M Tris-0.005 M $MgCl_2$ -0.001 M PMSF-0.01 M 2-mercaptoethanol, pH 7.6. Lysozyme was then added to a final concentration of 1 mg/ml and the suspension was incubated at 37°C with stirring. After 1 hr, lysis had occurred and the viscous mass was spun

mass was spun at 12,000 g for 1 hr. The clarified extract was decanted and saved. The protein concentration was adjusted to 10 mg/ml with buffer if necessary.

The pH of the extract was adjusted to 6.0 with 1 N acetic acid. A protamine sulfate solution (10% w/v) was added to the extract with stirring until a final concentration of 2% protamine sulfate was achieved. After stirring for an additional half-hour, the precipitated nucleic acids were removed by centrifugation.

Solid ammonium sulfate was added to the extract to give an 80% saturated solution. The precipitate was collected by centrifugation and then dissolved in a minimal volume of buffer. The enzyme solution was dialyzed against 0.05 M Tris-0.005 M MgSO_4 -0.01 M 2-mercaptoethanol-0.4 M NaCl-20% glycerol, pH 7.6 (buffer A) overnight at 4°C. Any precipitate that formed during dialysis was removed by centrifugation.

The dialysate was applied to a 2.5 x 40 cm DEAE-Sephadex A-50 column which had been equilibrated with buffer A. After absorption, the column was washed with buffer A until the OD_{280} of the eluate was less than 0.1. The column was then developed with a linear gradient (500 ml each of buffer A and either 0.4 or 1.2 M NaCl) and 10 ml fractions were collected. Enolase activity eluted after a major peak at 0.8 M NaCl. Fractions containing

greater than 10% of the peak activity were pooled and concentrated by ultrafiltration.

Table 1 summarizes a typical purification scheme for enolase. The recovery was usually greater than 70% of the starting activity. Starting with 400 gm of washed packed cells yields of 10-30 mg of pure enolase were obtained.

Table 1
Purification Scheme for Enolase

Step	Specific Activity	Total Activity	% Yield	Purification
Crude Extract	0.95	5500	100	1
0-80% $(\text{NH}_4)_2\text{SO}_4$	1.00	5300	96	1
DEAE-Sephadex Pool	121	5100	93	127

Stability of Enolase During Storage

Enolase activity in crude extracts was stable for at least 2 days at 4°C or -20°C. However, the activity of purified enolase was rapidly lost unless the following measures were taken to stabilize the enzyme. It was essential that the purified enolase be stored at a

concentration greater than 1 mg/ml in buffer A. High ionic strength also seemed to have a stabilizing influence. When stored at these conditions listed above, enolase activity was stable for months at -20°C . Enolase stored as an ammonium sulfate slurry also retained its activity for several months at 4°C .

Purity of the Enolase Preparations

Polyacrylamide gel electrophoresis of native purified enolase revealed only one band of protein stainable with Coomassie blue (Figure 1). In a duplicate gel, the position of enolase activity corresponded to the protein band. When the enolase preparation was subjected to SDS gel electrophoresis, only one band of protein was observed (Figure 2).

If the previously mentioned precautions for optimal stability were not observed, non-denaturing gel electrophoresis yielded a pattern with 2 major bands, one of which migrated with a mobility identical to homogeneous enolase, both of which had enolase activity. However, on SDS gels, only 1 protein band was observed migrating at a position identical to that of homogeneous enolase.

Figure 1. Densitometric scan of pure enolase.

Approximately 20 μ g of pure enolase was subjected to electrophoresis on 7% gels according to the method of Davis (11). Following staining with Coomassie blue and destaining in 10% acetic acid - 25% methanol, the gel was placed in a Gilford gel scanner and scanned at 600 nm.

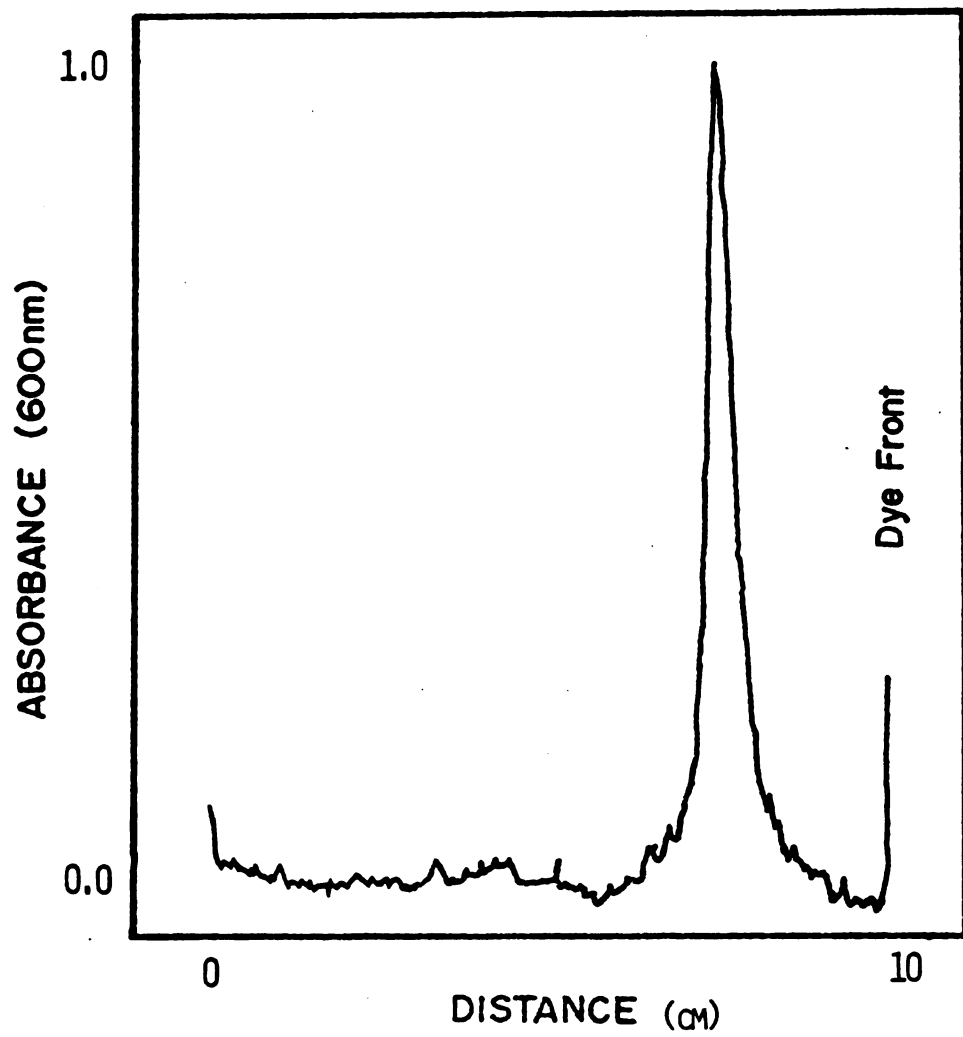
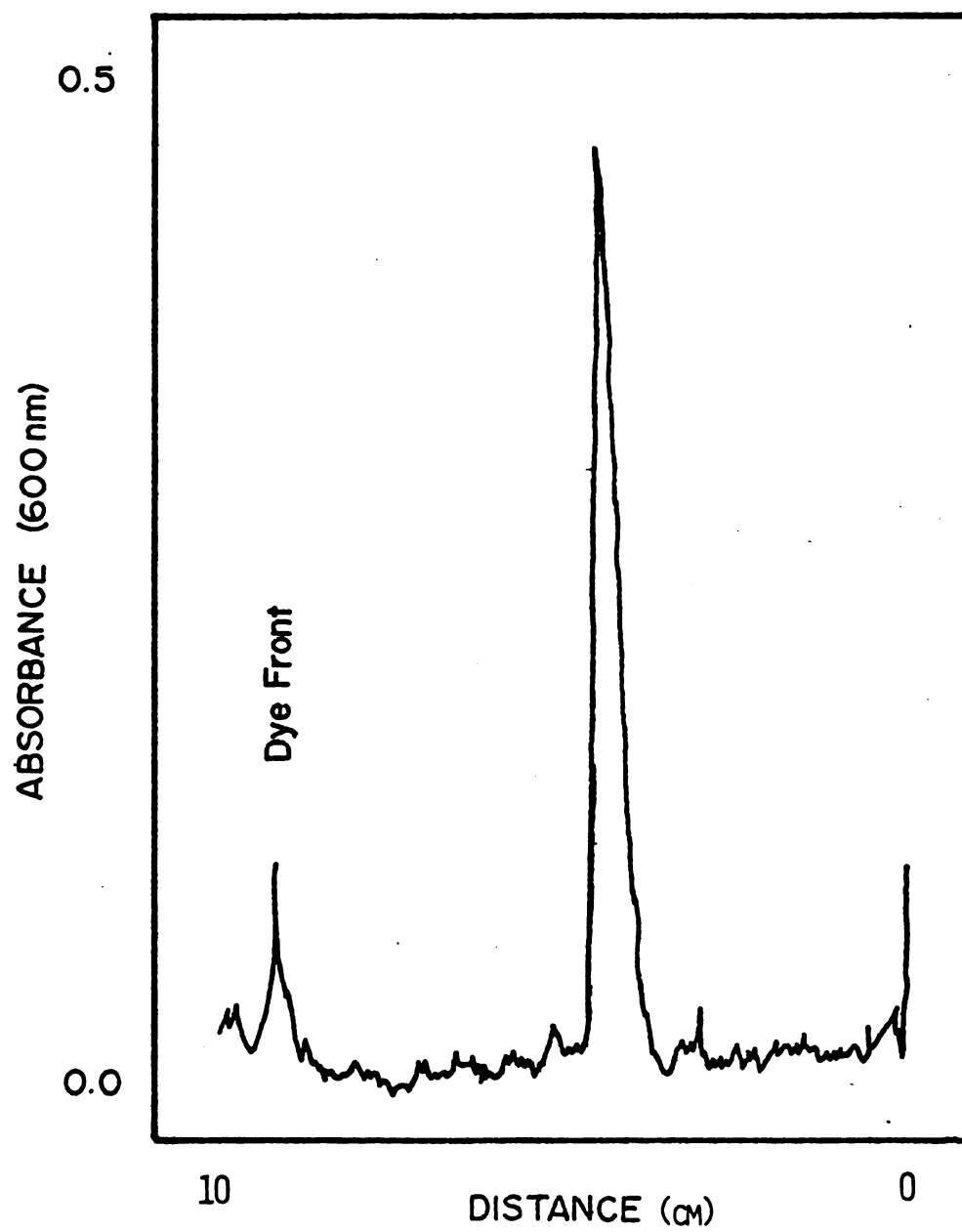


Figure 2. Densitometric scan of purified enolase.

Approximately 20 μ g of pure enolase was denatured and subjected to SDS gel electrophoresis on 8.75% gels according to Laemmli (30). After staining with Coomassie blue and destaining, the gel was scanned in a Gilford gel scanner at 600 nm.



Kinetic Properties of Enolase

Enolase from B. licheniformis exhibited typical Michaelis-Menton saturation kinetics with 2 PGA as substrate. Using either crude or purified enzyme, the K_m for 2 PGA was 0.4 mM. Under typical assay conditions, the V_{max} was 100 units/mg. Assuming that the average specific activity for enolase is 1 unit/mg, calculations show that enolase constitutes 1% of the cell's soluble protein.

The pH optimum for enolase was 8.0 in 0.05 M Tris-HCl buffer. At pH 6.5 the enzyme was only one-fifth as active as at pH 8.0. The enzyme was equally active in either 0.05 M Tris or 0.05 M KPO_4 at pH 8.0.

Enolase activity, in the presence of phosphate, is sensitive to the fluoride ion. Inclusion of 1 mM F^- and 5 mM K^+ phosphate in the assay completely inhibited enolase activity. The addition of chelating agents, such as 10 mM EDTA, also completely inhibited enolase activity.

A number of small molecules were tested for their ability to affect enolase activity. The presence of 5 mM AMP, ADP, ATP, PEP, or phosphoglycolate in the assay mixture did not produce a significant degree of inhibition or activation of enolase activity.

Molecular Weight of Enolase

The molecular weight of enolase in crude extracts was determined using the sedimentation velocity method of Martin and Ames (33). Using catalase and urease as internal standards, the molecular weight of the native enolase was calculated to be 370,000 daltons.

Following dissociation of purified enolase in SDS and 2-mercaptoethanol at 95°C, gel electrophoresis yielded a single protein band with a molecular weight of 47,000. Thus, the combined data (sedimentation and gel electrophoresis) suggest that enolase is an octamer comprised of 8 identical subunits.

Production of Antiserum

In order to properly study the fate of enolase protein during growth and sporulation, it was necessary to have a means of specifically isolating enolase from a crude extract. Specific precipitation of enolase with anti-enolase antibody seemed to be the best way to achieve the necessary isolation of the enzyme.

Highly purified enolase was used in the production of monospecific antiserum. Employing the following criteria, the antiserum obtained was shown to specifically precipitate enolase from crude extracts.

Ouchterlony double diffusion. Only one band of cross reacting material was observed when either a crude

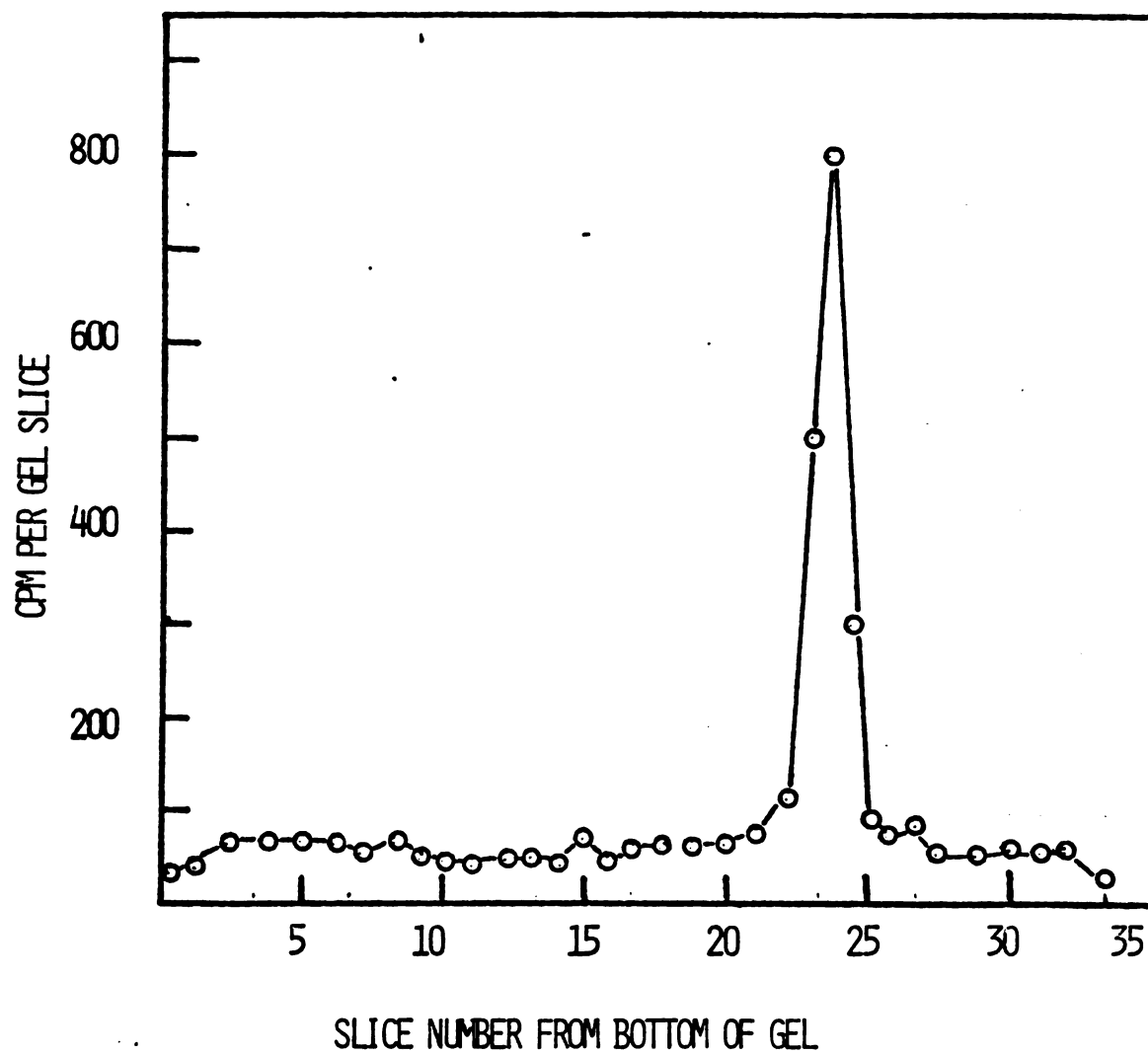
extract or purified enzyme was employed as the antigen. Fusion of these bands suggest an identity reaction.

Immunoelectrophoresis. Only one precipitin arc was observed after electrophoresis and immunodiffusion when either crude or purified enzyme was used.

SDS gel electrophoresis of an immunoprecipitate from a labeled cell extract. A cell free extract was prepared from cells which had been grown for several generations in the presence of 0.05 $\mu\text{Ci/ml}$ of $\text{U-}^{14}\text{C}$ -leucine. The cells were disrupted and a volume of extract containing 20 units of enolase was treated with an excess of anti-enolase serum overnight at 4°C. The precipitate that was formed was collected by centrifugation and was washed 3 times with 0.15 M NaCl. One hundred microliters of a solution containing 10 mM Na^+ -phosphate-1% SDS-10% 2-mercaptoethanol-0.002% Bromphenol blue-10% glycerol was added to the precipitate and was heated for 20 min at 95°-100°C. The denatured protein was layered onto a polyacrylamide gel containing 0.1% SDS which was then subjected to electrophoresis as previously described. After electrophoresis, the gel was cut into 2.5 mm slices, dissolved in H_2O_2 , and counted. The mobility of the single peak of radioactivity corresponded to the mobility of the enolase subunit, mw 47,000 (Figure 3). The above results suggest that the antibody obtained can specifically precipitate enolase

Figure 3. SDS gel electrophoresis of a ^{14}C labeled immunoprecipitate.

Cells were labeled in vivo with ^{14}C -leucine and the enolase was precipitated from extracts with antibody. After washing, the precipitate was prepared and subjected to electrophoresis in 10% gels by the method of Weber and Osborn (64).



from a crude extract.

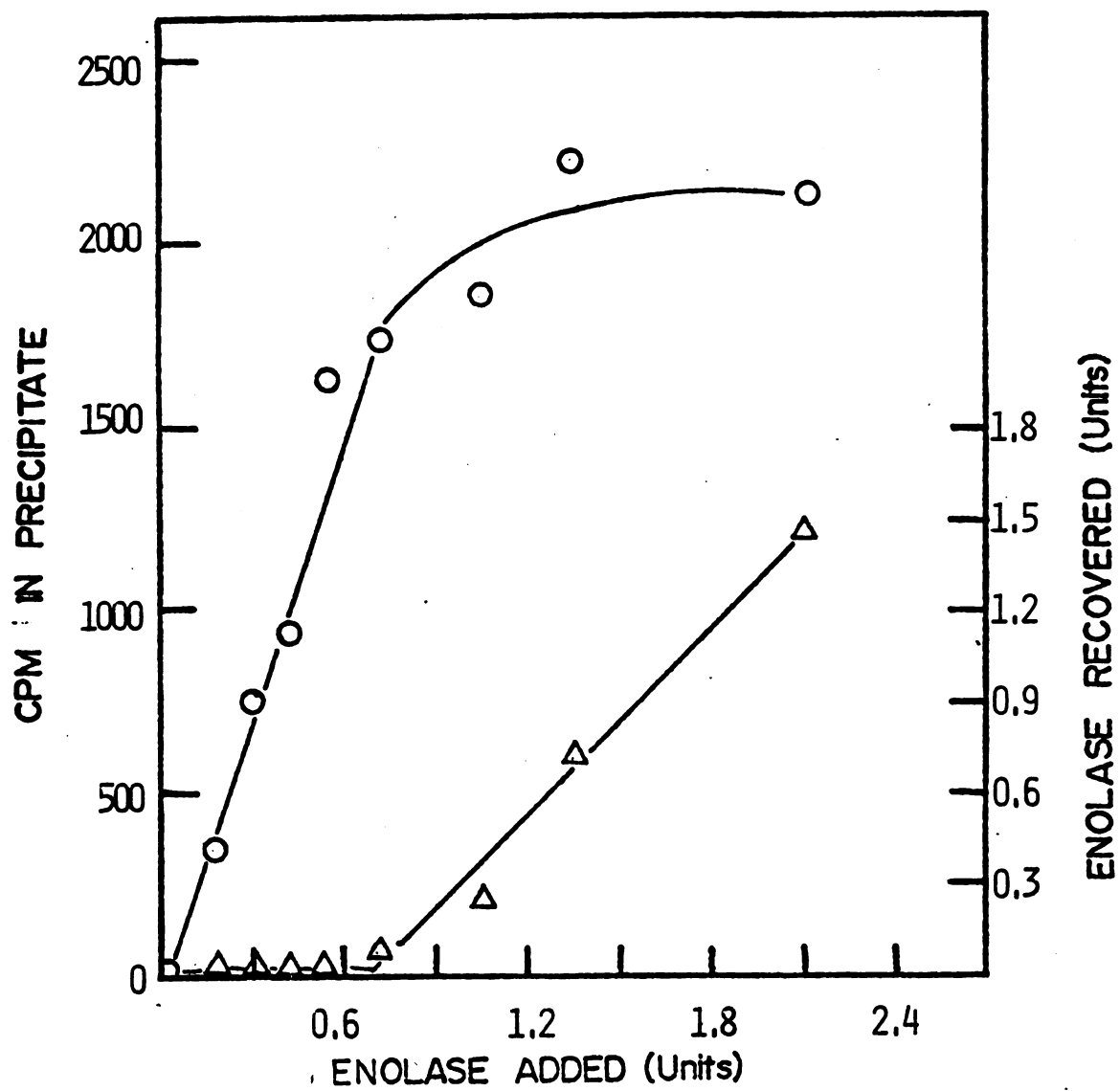
Quantitative precipitation of enolase with antibody was also demonstrated. Figure 4 shows that the precipitation of enolase from a ^{14}C -leucine labeled extract was quantitative based on the amount of radioactivity in the precipitate and by the quantitative recovery of enzyme activity beyond the equivalence point. Control serum did not affect enzyme activity nor did it precipitate a great deal of protein. During the course of the incubations, some protein did precipitate and therefore controls were always included to account for this non-specific precipitation.

Efficiency of the Precipitation Reaction

Precipitation of enolase with antibody is quantitative as shown in the preceding section. The efficiency of the precipitation approached 100% and quantitative precipitation was obtained with as little as 0.1 units of enolase. Since enolase has a molecular weight of 370,000 and an average V_{max} of 100 units/mg (corresponding to a turnover number of 37,000 moles PEP formed/mole of enolase), it can be shown that 0.1 unit of enzyme corresponds to 1 μg of protein. Because the quantity of enolase used in most of the experiments was 5 to 10 times greater than this amount, the addition of a known amount of carrier enolase was omitted.

Figure 4. Quantitative precipitation of enolase.

Increasing amounts of a ^{14}C -leucine labeled crude extract were added to tubes containing a constant amount of antibody. After overnight incubation at 4°C , the radioactivity in the precipitates and the residual enolase activity in the supernatant fluids were determined.



Profile of Enolase Activity During Growth and Sporulation in a Minimal Medium

The specific activity of enolase in crude extracts remained constant throughout growth and early sporulation averaging 1.37 units/mg (Figure 5). The amount of whole cell protein also remained constant during sporulation (about 1 μ g protein/ml Klett unit) but some cell lysis occurred beyond T_8 as evidenced by a reduction in culture turbidity and by examination by phase microscopy. Since the specific activity of enolase and whole cell protein (mg/ml culture) remain constant during sporulation, the amount of enolase present (units/ml) must also remain constant.

Titration of the Enolase Protein During Sporulation

Since the activity of enolase remained constant during post exponential growth, it was of interest to determine the relationship of the enolase activity to enolase protein. This yielded data concerning the intrinsic specific activity of the enolase molecule and the possible accumulation of enzymatically inactive enolase molecules.

Activity neutralization using the monospecific anti-enolase serum was employed to determine the amount of enolase protein in the crude extracts. The results of such experiments are shown in Table 2 and suggest that

Figure 5. Specific activity profile during growth and sporulation in A medium.

B. licheniformis was grown in 15 mM glucose-A medium. Samples were withdrawn and cell free extracts were prepared. Enolase activity was measured as described in Materials and Methods.

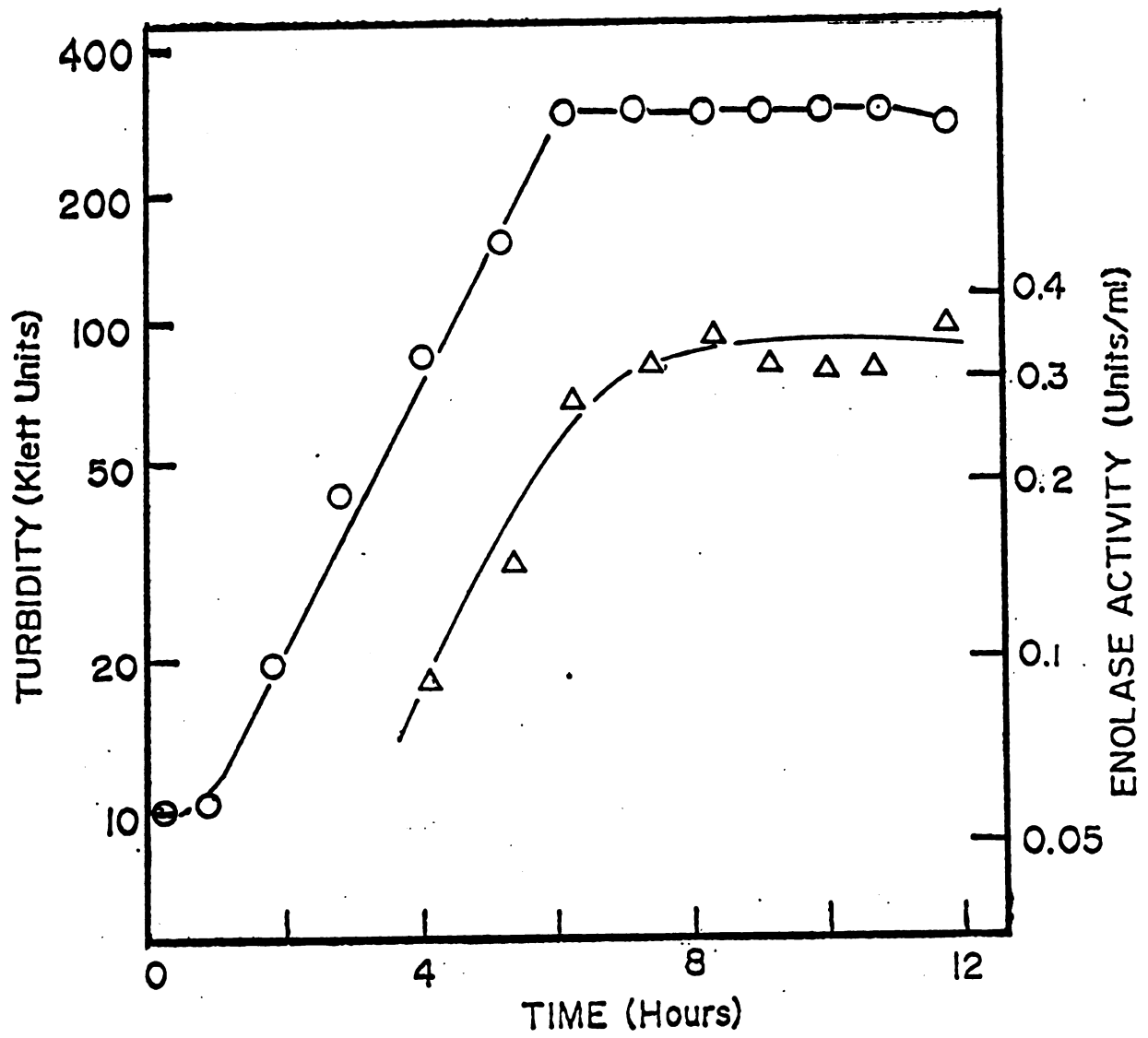


Table 2
Activity Neutralization of Enolase
in Crude Extracts

Time of Sample	Volume of Antibody Required to Inhibit 1 Unit of Enolase (μ l)
T ₀	11 (100)
T ₂	12.5 (113)
T ₄	13 (118)
T ₇	12.3 (111)

the amount of enolase activity is proportional to the amount of enolase protein and that very little (if any) enzymatically inactive cross reactive protein is present in the extracts. In other experiments where radioactively labeled immunoprecipitates were subjected to SDS polyacrylamide gel electrophoresis, only one band of radioactivity (with identical mobility) was observed regardless of the time of growth from which the enolase was isolated.

Protein Turnover During Sporulation

Using a pulse-chase technique to measure protein turnover, it was demonstrated that 2 to 3 hrs after the cessation of exponential growth, the rate of protein turnover increased from less than $3\% \text{ hr}^{-1}$ to $13\% \text{ hr}^{-1}$. This rate of loss of TCA insoluble radioactivity continued until at least T_8 (and possibly longer) (Figure 6). The addition of chloramphenicol (100 $\mu\text{g/ml}$) to the culture at T_0 completely inhibited protein turnover.

Fate of Enolase During Sporulation

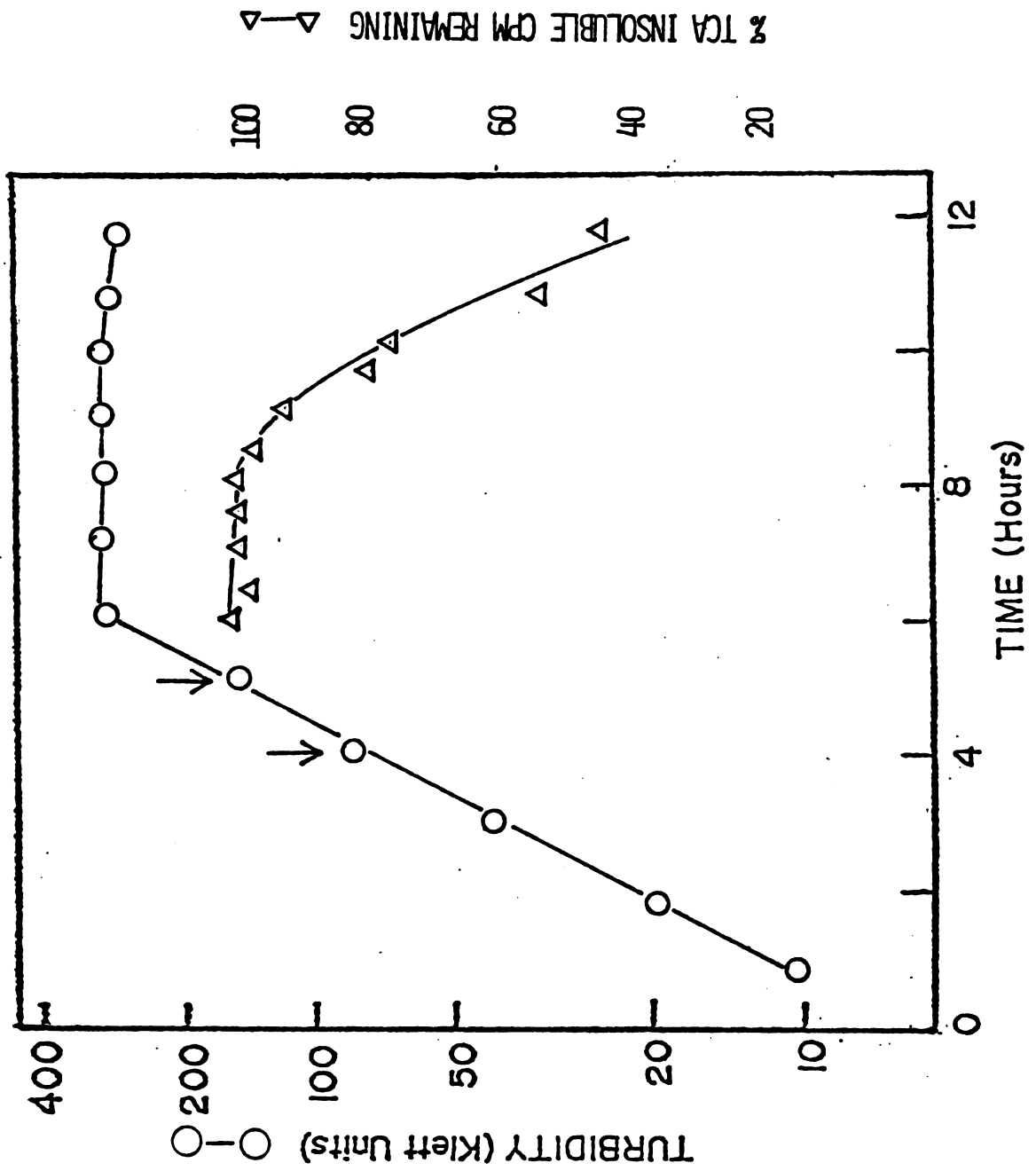
Since enolase activity remained constant throughout early sporulation, it was of interest to determine whether enolase was resistant to proteolytic attack or if the amount of enolase present represented a balance between synthesis and degradation.

In order to distinguish between the two possibilities, a double label experiment was designed to measure the rate of enolase degradation relative to general protein turnover (Technique 1, page 28). An extract labeled with ^{14}C -leucine served as an internal reference standard; the extracts from the culture labeled with ^3H -leucine provided a measure of the degradation of enolase. A quotient was obtained by dividing the $^3\text{H}:^{14}\text{C}$ ratio in total protein (A) by

Figure 6. Protein turnover during sporulation in A medium.

Cells growing in A medium were pulsed with 0.1 Ci/ml of ^3H -leucine at T_{-2} . At T_{-1} unlabeled leucine was added to a final concentration of 4 mM. Samples were taken and the TCA insoluble counts were determined. The initial value obtained was set equal to 100%.

The first arrow indicates the time of the pulse and the second arrow indicates the chase.



the $^3\text{H}:^{14}\text{C}$ ratio in enolase (B). If enolase were stable to proteolytic attack, the value of A/B would decrease due to a loss of total protein ^3H counts because of protein turnover at a rate of $13\% \text{ hr}^{-1}$. Theoretical decay curves for $0\%/ \text{hr}$ and $13\%/ \text{hr}$ turnover of enolase are shown in Figure 7 along with experimentally derived values of the function A/B. It can be seen that the experimental values fall along the theoretical 0% turnover curve until T_4 . After T_4 , the changes in the ratios become small, suggesting that enolase may be degraded. However, this method lacks sufficient sensitivity to determine precise rates of degradation at these later times.

An alternative method was therefore used to determine the turnover of enolase. The enzyme was quantitatively precipitated from crude extracts and the specific radioactivity (cpm/unit) was determined. The rate of enolase degradation could then be measured by plotting the change of specific radioactivity versus time.

When this type of experiment was performed, very curious results were obtained (Table 3). The specific radioactivity remained constant until about T_4 at which time the specific radioactivity began to increase. This experiment was repeated several

Figure 7. Enolase turnover measured by the ratios method.

Two cultures of B. licheniformis were grown in 15 mM glucose-A medium, pulsed with either ^{14}C - or ^3H -leucine, sampled and manipulated as described in Materials and Methods. The theoretical curves for 13%/hr turnover (Δ - Δ - Δ) and 0%/hr turnover (0-0-0) are plotted. We assume that turnover begins between T_1 and T_2 . The experimental points (0-0-0) shown are the averages of three experiments.

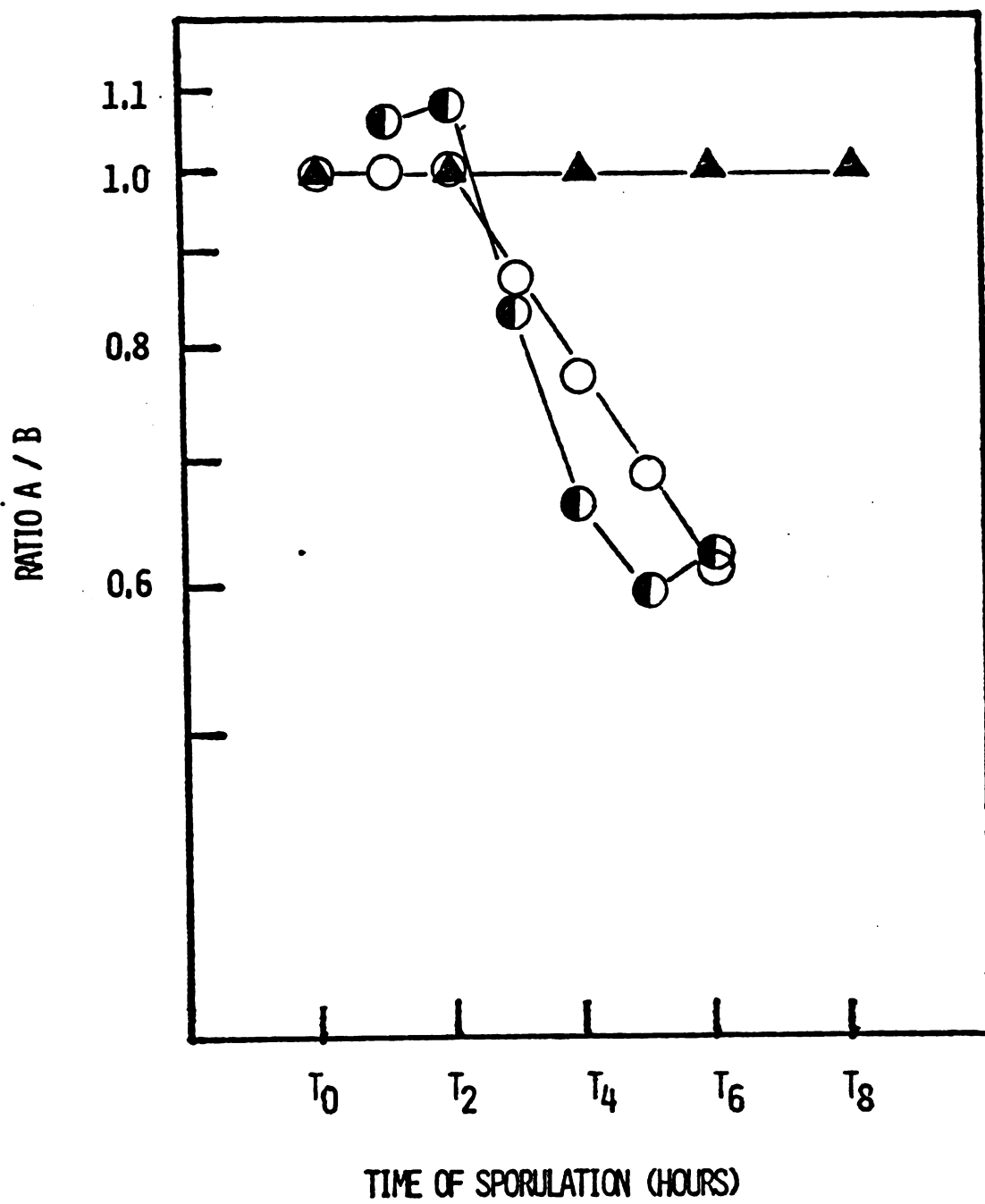


Table 3
Results of the Quantitative Precipitin Tests

Time of Sample	Specific Radioactivity (CPM/unit of enolase)
T ₀	2877 (100)
T ₁	2586 (90)
T ₂	2723 (95)
T ₃	3098 (107)
T ₄	3131 (108)
T ₅	3301 (115)

times with the same results. This suggests that the chase of unlabeled leucine was not sufficient to prevent reutilization of labeled leucine and that enolase synthesis occurred during sporulation.

Another type of experiment was designed to measure the turnover of enolase. This approach took advantage of the constant amount of enolase protein. If enolase was degraded during sporulation, then an equal amount of enolase would have to be synthesized in order to maintain enolase at a constant level. This experimental design involved pulse-labeling the

cells for short (20 min) periods of time with ^3H -leucine at various times during growth and sporulation followed by immunoprecipitation of the enolase from the crude extracts. When this type of experiment was performed, label was detectable in all of the immunoprecipitates regardless of the time from which they were prepared. This indicates that enolase synthesis occurs during post-exponential growth and implies that some degradation of enolase has occurred.

Profile of Sporulation Related Events in Minimal Media

During the course of the experiments conducted in minimal media, it was noted that the sporulation process proceeded very slowly. Refractile bodies and heat resistant spores did not appear in the culture until T_{24} . In addition, the efficiency of sporulation was very low; only 25% of the cells present at T_0 ultimately yielded spores. Although synthesis of extracellular protease (data not shown) increased at T_2 - T_3 , it was concluded that although the initiation and early events of sporulation proceeded normally, sporulation was arrested at stage 2 and proceeded very slowly and inefficiently. Therefore, this medium was not suitable for studies of the fate of enolase during sporulation and further studies were conducted in SNB media.

Sporulation Pattern of *B. licheniformis* in SNB Medium

B. licheniformis sporulated well in SNB medium. Exponential growth ceases 2 to 3 hours after inoculation at about 150 KU upon the exhaustion of glucose from the medium. A drop in pH, usually observed during growth in most media (23), is not observed in SNB media due to the high buffering capacity of the media.

The turbidity continued to increase after the end of exponential growth for several hours. The amount of whole cell protein remained proportional to the turbidity throughout growth and sporulation. A value of 1.22 μ g of protein per ml-KU was calculated for the cell protein during growth.

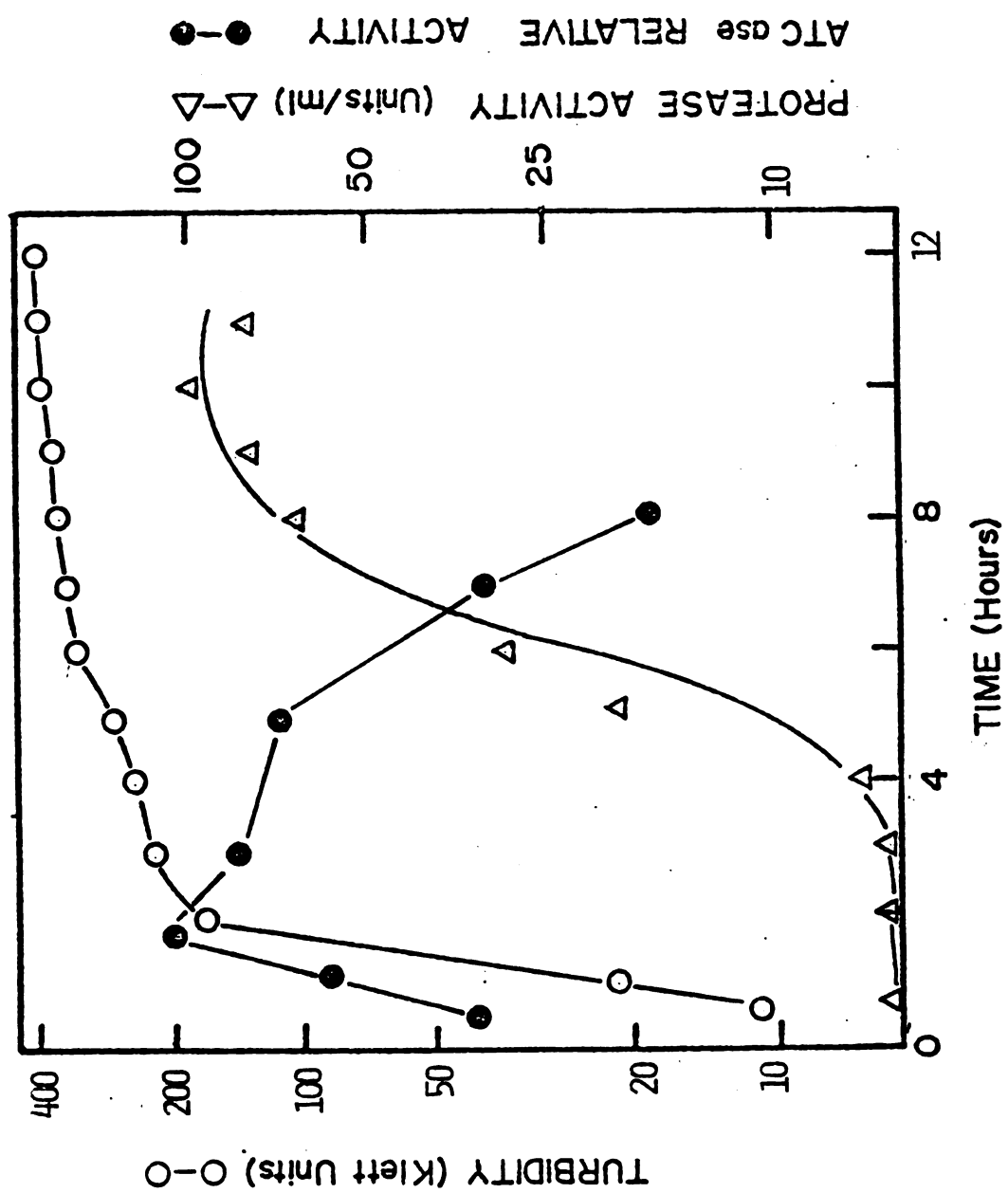
Refractile bodies were first observed at T_8 - T_9 using phase contrast microscopy and heat resistant spores began to appear at T_9 - T_{10} . Sporulation was essentially complete at T_{12} .

The profiles of several enzymes associated with sporulation were examined. Extracellular protease activity increased dramatically around T_2 while ATCase activity decreased rapidly with a half-life of approximately 2 hours (Figure 8).

The above results indicate that *B. licheniformis* resembles other sporulating bacilli with respect to

Figure 8. Profile of ATCase and extracellular protease activity during sporulation.

Cells were grown in SNB and samples were withdrawn at various times. Enzyme assays are described in the Materials and Methods.



the timing of sporulation and various enzyme activities.

Profile of Enolase Activity During Sporulation

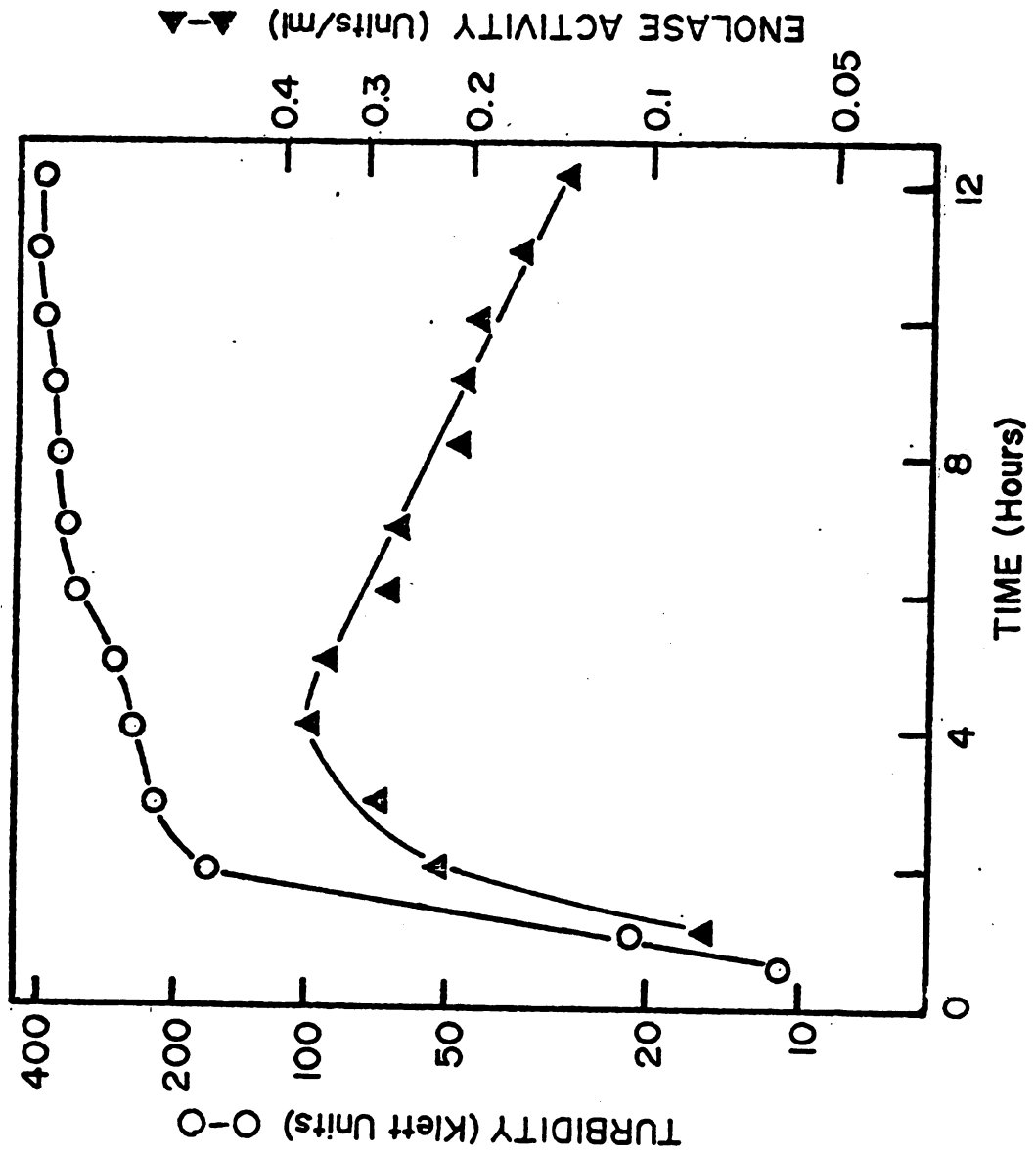
Enolase activity was measured in cell free extracts prepared from samples of cultures taken at various times during growth and sporulation. Figure 9 shows the profile of enolase activity during sporulation. Expressing the measurements of the enzyme in terms of specific activity can be misleading in quantitating changes in the amount of enolase protein. Enolase activity peaked at T_2 and declined throughout sporulation. The half-life of the enzyme activity was about 5 hr and the rate of decay was fairly constant throughout sporulation. Enolase activity was difficult to accurately measure due to difficulties in obtaining consistent breakage of the spores. However, an average specific activity of 0.1 units/mg was obtained in several experiments.

The Fate of Enolase During Sporulation. Consideration of the Various Possibilities

The data presented thus far indicate that enolase activity decreases during sporulation. One or more of the following events could explain the loss of enolase activity: (1) Excretion of the enzyme into the culture fluid, (2) Compartmentalization, (3) Covalent modification, (4) Modulation of enzyme activity by effector

Figure 9. Enolase activity during growth and sporulation.

A 1 liter culture (SNB medium) in a 3 liter Fernbach flask was incubated at 37°C with vigorous aeration (250 rpm). 25 ml samples were withdrawn at various times and extracts were prepared. Enolase activity was measured as described previously.



molecules, (5) Instability of the enzyme in crude extracts, (6) Degradation of the enzyme. The following experiments were performed in order to determine the mechanism of the disappearance of enolase activity.

Excretion of the enzyme during sporulation could be tested in two ways. The first simply required assaying an aliquot of the culture fluid for enolase activity. No enolase activity was detected in the culture fluid at any stage of growth or sporulation. An assay for enolase protein was made using Ouchterlony double diffusion analysis. A 1 ml sample of culture was clarified by centrifugation and made 1 mM in PMSF. This protease inhibitor was used to inhibit the very active extracellular proteases secreted during sporulation. A sample of this treated culture fluid was used in one of the wells on an Ouchterlony slide in opposition to anti-enolase serum. After overnight incubation in a moist atmosphere, precipitin lines were not visible in any of the samples.

Compartmentalization of enolase (and hence its disappearance) could be ruled out since, upon microscopic examination, it was noted that greater than 90% of the cells were broken by the sonication treatment used to prepare the cell extract and no regular organelle-like structures were present.

The modification of enzyme activity by covalent modification is well documented (49). Glutamine synthetase is probably the best known example and involves the attachment of an adenylyl group to the subunit via a tyrosyl hydroxyl group (50). Modification of proteins can lead to altered kinetics (28), altered mobility on gels (20), or altered spectral data (50). Enolase isolated from late exponential phase cells and from sporulating cells have identical K_m 's for 2-PGA (0.44 mM) and identical mobilities on polyacrylamide gels. Spectral data were difficult to compare since the preparations often contained some minor nucleic acid contamination which absorbed strongly in the 260 nm region. However, no differences in the spectrum above 300 nm could be detected.

It was possible that some molecule (large or small) was inhibiting enolase activity in sporulating cells. One way to test for an inhibiting factor in cell extracts is to perform mixing experiments. In this kind of experiment, crude cell extracts were prepared from cells harvested at T_{-1} and T_4 . Enolase activities in the separate extracts were assayed and then portions of the extracts were mixed, incubated for 10 min at 30°C and assayed. The two samples contained 0.16 units/ml and 0.32 units/ml and, after mixing equal portions of

the extracts, a value of 0.24 units/ml was observed for the mixture. In a different kind of experiment, an extract of sporulating cells was assayed before and after overnight dialysis against 500 volumes of 50 mM Tris-5 mM MgCl_2 -20% glycerol-10 mM 2-mercaptoethanol, pH 7.6. The activity was unchanged by dialysis.

The stability of enolase in crude extracts was examined in order to assess whether or not the loss of activity during sporulation was due to the instability of the enzyme. Very often the lack of a stabilizing ligand can affect the measured activity of an enzyme (38,61). Also, it was necessary to test the in vitro resistance of enolase to degradation by proteases. Deutscher and Kornberg have shown that the activities of several enzymes decreases when the extract is incubated for a prolonged period of time at 37°C (12).

B. licheniformis was grown in SNB medium. A sample of the culture was taken during exponential growth (T_1) and another sample was taken during sporulation (T_5). In this type of medium, protein turnover had begun and the decrease of enolase activity was underway at the time the second sample was withdrawn. Extracts were prepared from each sample and incubated at either 4°C or at 37°C. Ten microliter

samples of extract were withdrawn over a 23 hour period and immediately assayed. Table 4 shows that enolase activity remained constant in all for at least 5 hours of incubation. The results suggest that enolase is stable in crude extracts. The data did not change when PMSF (1 mM) was omitted from the extract buffer.

Table 4
Stability of Enolase in Crude Extracts

Time of Sample	% Activity Remaining			
	T ₋₁		T ₅	
Incubation Time	4°	37°	4°	37°
0 hrs	100	100	100	100
1 hrs	ND	100	ND	125
2 hrs	ND	100	ND	107
5 hrs	ND	100	ND	72
6 hrs	ND	100	ND	68
8 hrs	ND	100	ND	68
23 hrs	100	ND	100	40

The decrease in enolase activity during sporulation in SNB could have been the result of degradation. Experiments were designed to demonstrate that a) the enolase activity could be correlated with enolase protein and b) yield data on the possible accumulation of inactive enzyme fragments. In these experiments, cell free extracts were prepared and a known amount of enolase activity (determined by assaying each extract) was added to tubes containing increasing amounts of antienolase serum. After incubation overnight at 4°C, the sample was spun in a centrifuge and the supernatant fluid was assayed for residual enolase activity. Table 5 shows the results of these experiments. These data indicate that the same quantity of antibody is required to inactivate one unit of enolase activity regardless of the time of sporulation at which the enzyme was obtained. This implies that a direct correlation exists between enolase activity and the amount of enolase protein present during sporulation. This also suggests that no inactive cross reacting material accumulates in the cell during enolase degradation.

Table 5
Activity Neutralization in Crude Extracts

Time of Sample	Amount of Antibody Required to Inhibit 1 Unit of Enolase (μ l)
T ₀	17.5
T ₁	18
T ₅	16

The Relationship of Protein Turnover to Enolase Activity

Having established that enolase activity and the amount of enolase protein decrease during sporulation, it was of interest to determine the relationship between protein turnover and enolase degradation.

Although protein turnover has been demonstrated during sporulation of B. licheniformis in minimal medium it could not be consistently demonstrated in a complex medium (SNB). Other investigators have been able to demonstrate turnover in a complex media in B. subtilis and B. cereus (8,56). A set of conditions was finally established where protein turnover could be consistently demonstrated (see Materials and Methods).

The rate of loss of TCA insoluble material was graphically determined and the degradation rate constant (K_d) was then converted to a $\%$ hr^{-1} value. The observed value of $10\% \text{ hr}^{-1}$ is well within the expected range of values reported in other Bacillus species (13). The increase in the rate of turnover began at T_2 which also corresponds to the initiation of the event in other Bacillus species. This is also the time at which enolase activity begins to decrease at much the same rate.

The Effects of Chloramphenicol and 2,4-Dinitrophenol on the Loss of Enolase Activity

The antibiotic chloramphenicol has been shown to inhibit the incorporation of amino acids into protein (31). The addition of chloramphenicol (100 $\mu\text{g/ml}$) to a culture of B. licheniformis at T_0 resulted in an apparant stabilization of the amount of enolase activity (Figure 10). The activity remained constant throughout the time course of the experiment. This result suggests that the development of a sporulation associated enzyme(s) is required for enolase degradation.

Inhibitors of energy metabolism have a profound affect on general protein turnover during sporulation (19). The nature of this energy requirement is unknown, but if the energy generation of the cell is

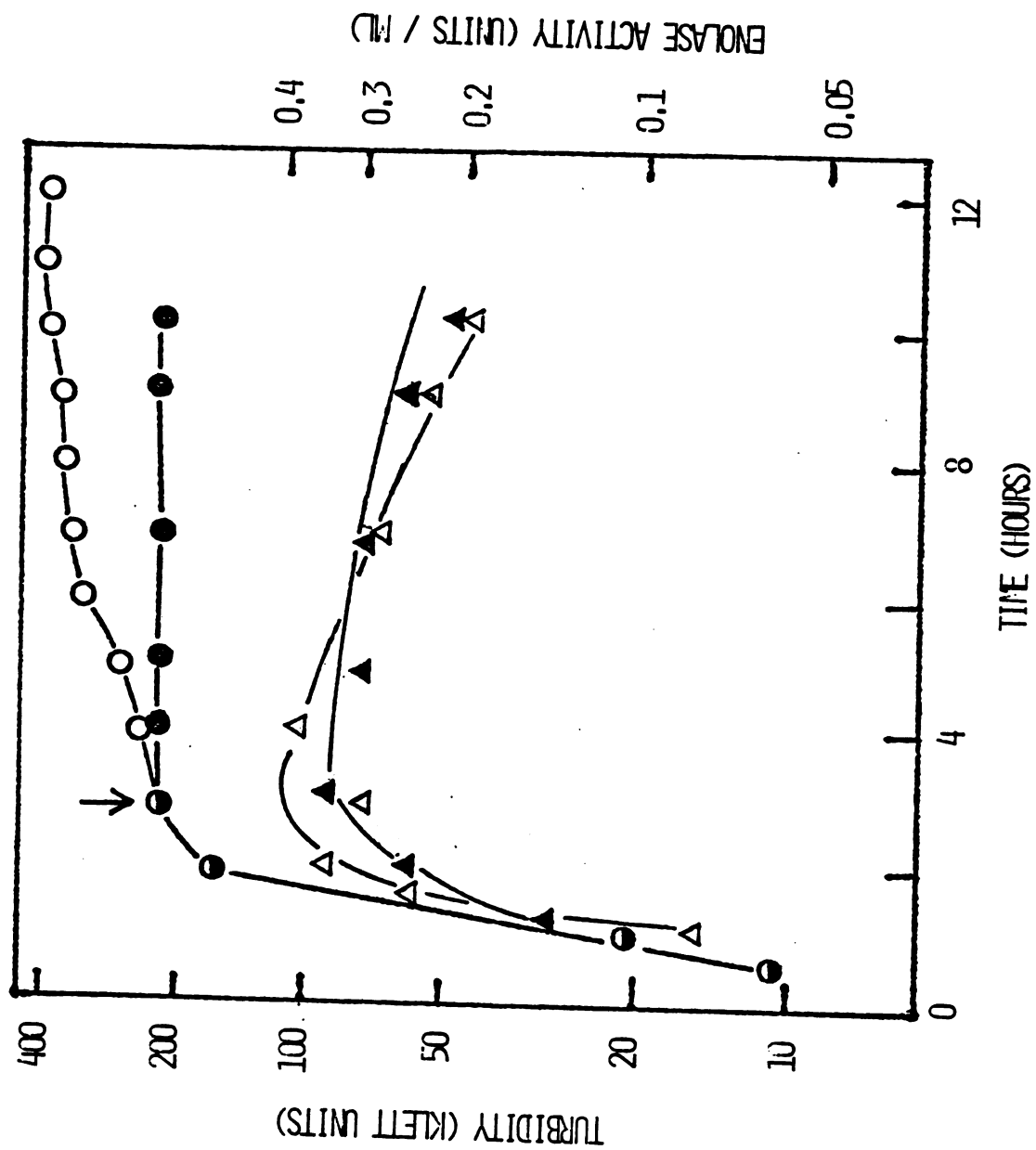
Figure 10. The effect of the addition of chloramphenicol on enolase degradation.

Two parallel cultures were grown in SNB medium under the standard conditions.

At T_0 , chloramphenicol (100 $\mu\text{g/ml}$) was added to one of the cultures. Samples were collected and harvested. Extracts were prepared and enolase activity was determined.

The open circles represent growth of the untreated culture while the closed circles represent growth of the treated culture.

The open and closed triangles represent enolase activity in the untreated and treated cultures, respectively.



interrupted, there is a decrease in the rate of protein turnover.

When 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, was added to a sporulating culture at T_3 , the decline in the amount of enolase activity was substantially reduced.

These results suggest that a correlation exists between general protein turnover, intracellular serine protease activity, and decreased enolase activity during sporulation.

Relationship of Enolase Degradation to Sporulation

Although enolase activity declines during sporulation there is no evidence to suggest that the event is sporulation specific or obligatory. The correlation of enolase degradation and the induction of protein turnover, however, suggest that these two events may be related.

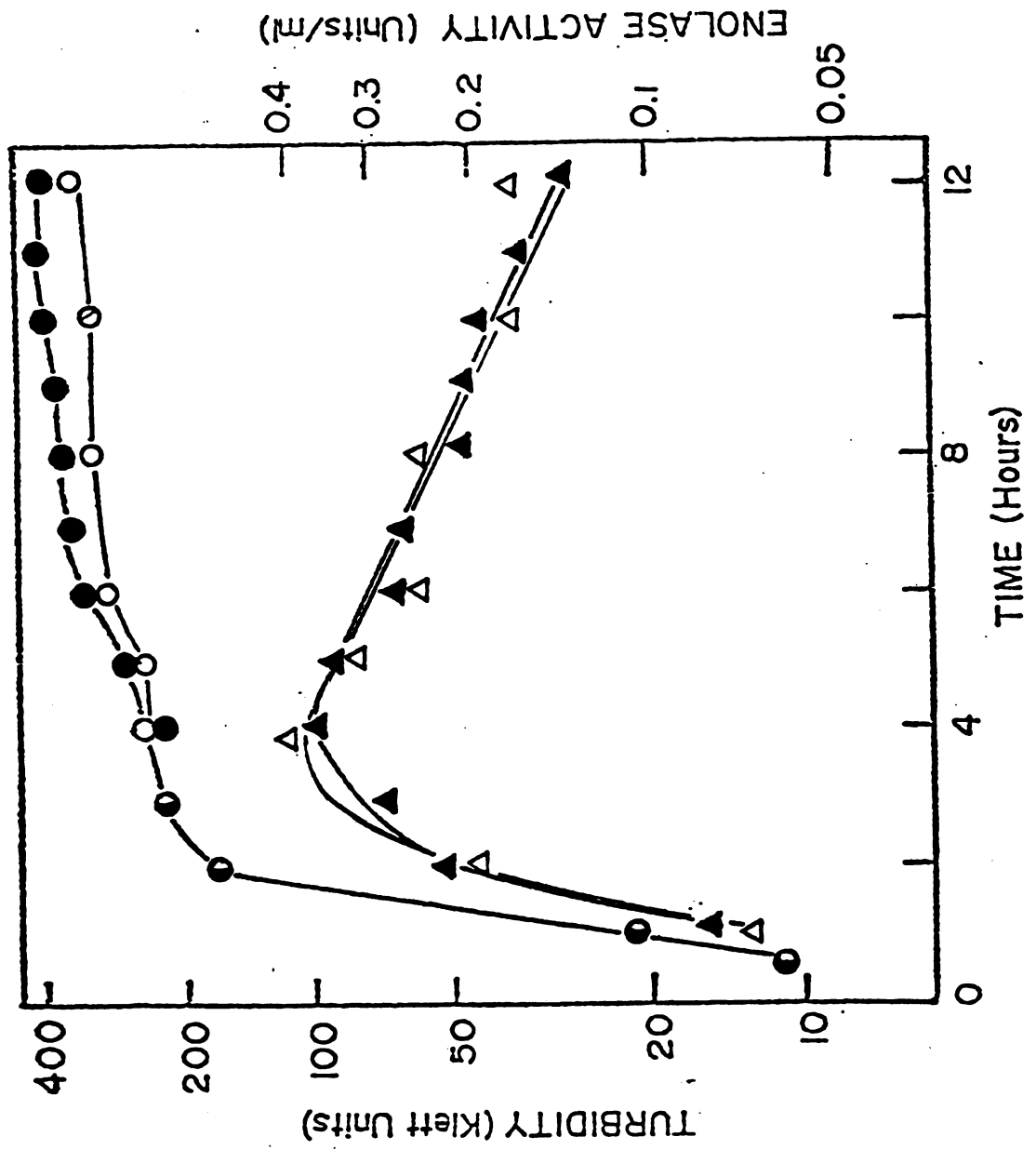
A thorough examination of the relationship of enolase degradation to sporulation could not be conducted in this study due to the lack of asporogenous mutants in B. licheniformis. An alternative approach was taken. Various compounds, when added to a sporulating culture, have been shown to repress sporulation (18). One such inhibitor, m-aminophenylbenzeneboronic acid (ABBA), has been shown to repress

the synthesis of the intracellular protease activity and repress sporulation in B. subtilis. Vegetative growth is unaffected by concentration up to 0.2 mM ABBA. Therefore, the compound appeared to be directed against sporulation specific events and was used in studying the relationship of sporulation to enolase degradation. m-Aminobenzeneboronic acid (final concentration - 0.2 mM) was added to a culture growing in SNB at T_{-1} . Samples were withdrawn at various times, extracts were prepared and enolase activity was determined. B. licheniformis responded to the addition of ABBA in a manner similar to that of B. subtilis. Refractile bodies were not detected even as late as T_{24} but extracellular protease activity increased although at a reduced rate and quantity. The effect of ABBA addition on the intracellular protease activity was not determined.

The decrease of enolase activity in cultures treated with ABBA was very similar to that observed in the control culture. The activity profile was identical to the control culture with respect to the timing and rate of enolase degradation (Figure 11).

Figure 11. The effect of the addition of ABBA on the rate of enolase degradation.

Two parallel cultures were grown in SNB as described in Figure 10. At T_{-1} , 2 ml of 0.1 M K^+ -ABBA was added to one of the cultures (final concentration: 0.2 mM). Samples were withdrawn and extracts were prepared as previously described and enolase activity was determined. The closed and open circles represent growth of the control and ABBA treated cultures, respectively. The closed and open triangles represent enolase activity in the control or treated cultures.



Enolase Synthesis During Sporulation

It has been stated that the amount of enolase protein present at any given time may represent a balance between its synthesis and degradation. The degradation rate can be approximated from the activity curve; however, this apparent rate constant does not take into account any synthesis of enolase that may occur during sporulation. Also, since it is difficult to prevent reincorporation of radioactive amino acids derived from proteolysis of prelabeled vegetative protein, the degradation of previously labeled enolase may be difficult to determine and interpret correctly.

The synthesis rate can be measured by a pulse technique using radioactive leucine. A sample of a culture was labeled for a designated time and then harvested. After preparation of a cell free extract, the enolase was precipitated with anti-enolase serum and the amount of radioactivity in the precipitate was determined. The radioactivity represented the amount of enolase synthesized during the labeling period. Changes in the specific radioactivity of the enolase at different periods of labeling were corrected by expressing the radioactivity in enolase as a percentage of the total label incorporated

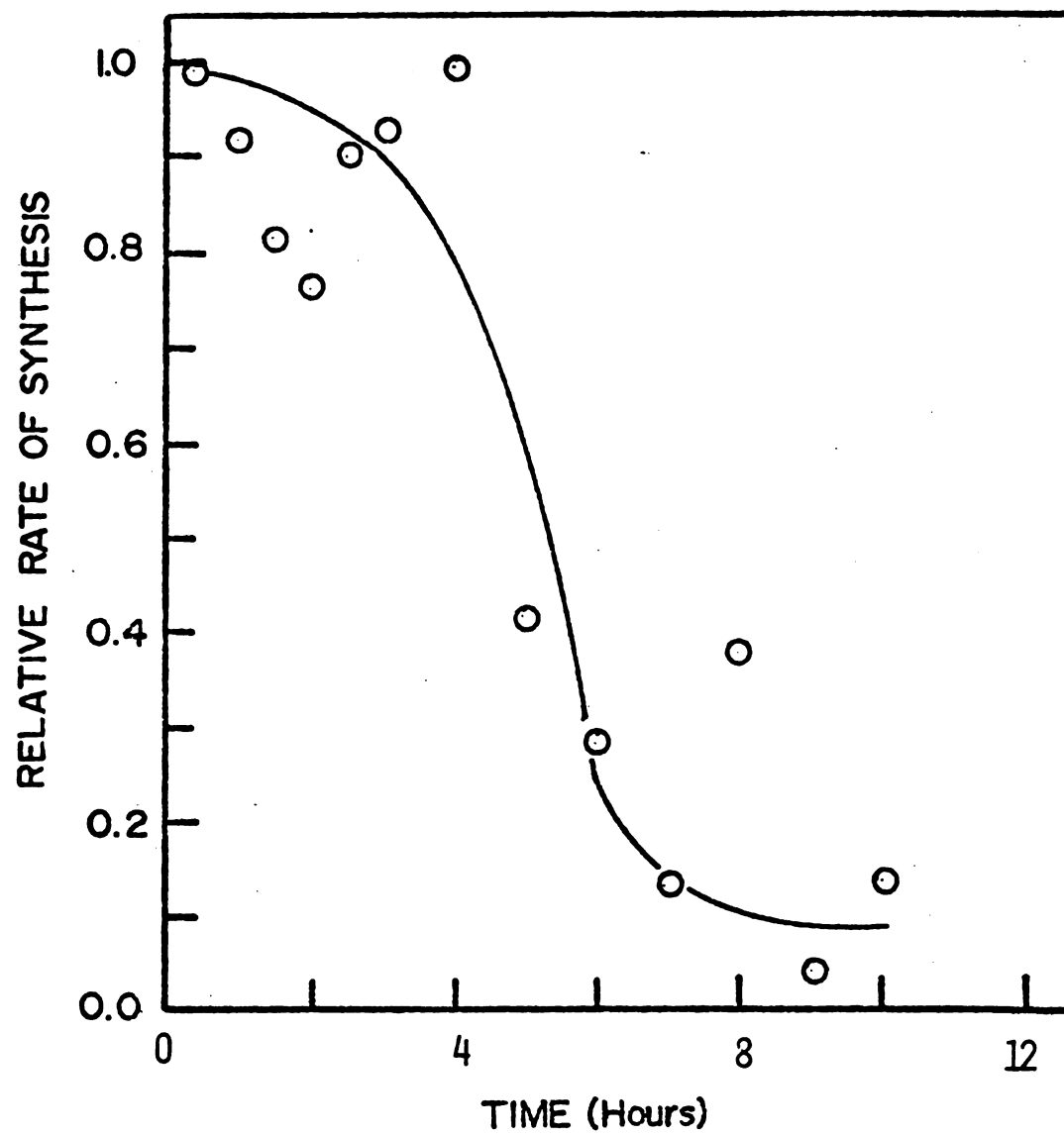
during the pulse.

When B. licheniformis was grown in SNB, the amount of label incorporated into enolase during exponential growth and early sporulation was approximately 1.5% of the total label incorporated. At T_3 , the rate at which label was incorporated into enolase decreased drastically. The rate of enolase synthesis decreased approximately 8-fold, from 1.5% of the total label/20 min to about 0.2% of the total label/20 min. The decrease in the synthesis rate was rapid and occurred between T_3 and T_4 . Enolase synthesis could be detected in sporulating cells as late as T_8 , the last sample examined.

Figure 12 illustrates the change in the rate of enolase synthesis during sporulation. The synthesis rate is expressed as a relative rate in order to better compare individual experiments. The maximum rate of enolase synthesis (% label in enolase/20 min) was assigned a value of 1.0 for purposes of comparison.

Figure 12. The synthesis rate of enolase during sporulation.

Cells were pulsed with ^3H -leucine and processed as described in Materials and Methods. The rate of synthesis (% label in enolase/20 min) has been normalized (based on the maximal rate equal to 1.0) to allow comparison of individual experiments.



DISCUSSION

Highly purified enolase has been prepared from B. licheniformis because relatively large amounts of homogenous enzyme were required for the production of enolase-specific antiserum. The enolase preparations obtained were judged to be pure by the criteria of gel electrophoresis of native and SDS-denatured protein and various immunochemical techniques.

Enolase isolated from B. licheniformis is very similar to the enolase from B. megaterium. Its physical properties (Table 6) are essentially identical to those for B. megaterium reported by Singh and Setlow (52). The discrepancy in the native and subunit molecular weights of the two enolases probably arises from the manner in which they were determined.

Antibody against B. licheniformis enolase cross reacts with enolases from B. megaterium, B. subtilis and B. cereus (data not shown). Precipitin bands on Ouchterlony diffusion slides partially fused, suggesting a great deal of intrageneric relatedness of the enolases.

Table 6
Comparison of Some Microbial Enolases

Property	<u>B. licheniformis</u>	<u>B. megaterium</u>	<u>Thermus Sp.</u>	<u>E. coli</u>	Yeast
Mol. Wgt.	370,000	335,000	382,000	92,000	88,000
Subunit Mol. Wgt.	47,000	42,000	48,600	46,000	44,000
KM 2-PGA	0.4 mM	0.71 mM	0.15 mM	0.10 mM	0.15 mM
Divalent Cation Requirement	Yes	Yes	Yes	Yes	Yes
F ⁻ Inhibition in Presence of P _i	Yes	Yes	ND	Yes	Yes
p ^H Optimum	8.0	7.8-8.3	8	8.1	7.7
Thermal Stability ¹ (A _{1/2})	80	ND	88-90	ND	55
Immunological Relatedness to <u>B. licheniformis</u>	--	Yes	ND	No	ND

¹A_{1/2} = The temperature at which the enzyme has a half life of 5 minutes.

Several general comments can be made concerning Bacillus enolases and other enolases from microbial sources. The enolase isolated from the bacilli seems to be more closely related to the enolase isolated from the genus Thermus than to the enolases from E. coli or yeast (55,64,65). The enolases from Bacillus and Thermus species share the same range in molecular weight and the octameric subunit structure while the E. coli and yeast enolases have a smaller molecular weight and a tetrameric subunit structure. The enolase isolated from the Thermus species is extremely heat resistant, an observation which is perhaps not too surprising since Thermus is an extreme thermophile (1). Enolase isolated from B. licheniformis is also surprisingly thermal stable. In 50 mM Tris or 50 mM K^+ phosphate buffer, pH 7.6, in the presence of 5 mM $MgCl_2$, the B. licheniformis enzyme has a half-life of 80 min at 60°C (data not shown). Yeast enolase, on the other hand, is much more heat labile ($t_{1/2}$ = 30 min (1)).

There seems to be several features common to all enolases of microbial origin. There is a requirement for Mg^{+2} for maximal activity and addition of the chelating agent, EDTA, inhibits enzyme activity. The specific activity of pure enolase and the K_m values

are fairly uniform for all the microbial enolases (1).

Enolase Metabolism During Growth and Sporulation in Minimal Medium

The specific activity of enolase remained constant throughout growth and sporulation in a minimal medium. Enolase activity (units/ml) also remained constant throughout sporulation.

Using a double label technique to measure enolase degradation, it was found that the enolase protein was fairly stable during growth and sporulation until T_4 . After T_4 , this technique proved to be too insensitive to measure degradation accurately. An alternative approach, that of quantitatively precipitating and determining the specific radioactivity of enolase, was employed to determine the extent of enolase degradation. Using this method, it was found that the specific radioactivity of enolase (cpm/unit) increased during sporulation. This was an unexpected result since it suggested that the chase procedure was not efficient in preventing reutilization of amino acids released by proteolysis. This same effect, the inability of exogenously added amino acids to exchange with the intracellular pools, has been previously observed (4). It is possible that this phenomenon is due to a loss of "exit permease" transport systems

during sporulation (4). Extending this observation, it is obvious that the observed rate of protein turnover in minimal medium is an inaccurate estimation of the true turnover rate. In fact, Bernlohr, using H_2^{18}O to measure protein turnover, concluded that B. licheniformis cells turnover 200% of their protein during sporulation (5).

Another type of double label experiment was designed to measure protein turnover. This technique took advantage of the fact that both total cell protein and enolase activity (and therefore protein) remained constant during early sporulation. Since incorporation of label into protein represented synthesis in balance with degradation, it was possible to compare the synthesis rate of enolase with enolase degradation. Using immunoprecipitation to isolate enolase, this type of pulse labeling experiment showed that label was incorporated into the enzyme.

Enolase degradation began at T_3 and the rate of degradation increased over the next 2 hours until it was 10%-12% hr^{-1} , a value close to the rate of general protein turnover.

I conclude from my studies that the amount of enolase present during early sporulation in A medium represents a balance between synthesis and degradation. That

is, the net change in enolase protein concentration is zero due to the balance of synthesis and the degradation.

Enolase Activity During Growth and Sporulation in SNB Medium

Enolase activity could be detected in cell free extracts prepared from all stages of growth and sporulation. This was to be expected since enolase is a key enzyme in glycolysis and in gluconeogenic metabolism.

Enolase activity (units/ml culture) reached a maximum value at about 3 hr after the onset of sporulation and thereafter decreased at a rate of $10\% \text{ hr}^{-1}$. Enolase activity could still be detected in extracts prepared from cultures as late as T_{10} and containing about 30% refractile bodies.

Evidence That Enolase is Degraded

It has been established that the amount of enolase activity decreases during sporulation. Excretion, of the enzyme, compartmentalization, inhibition and degradation, were all considered as alternative mechanisms to account for the decrease in enolase activity and protein.

Excretion of enolase into the culture fluid was ruled out as a means accounting for the loss of activity. Assay of the culture fluid either for enolase activity

or cross reacting material yielded negative results. Compartmentalization of the enzyme was ruled out since there were no regularly shaped structures in the broken cell suspension. Sonication resistant, forespore-like compartments would have been easily recognizable. A thorough immunological search for enolase protein (either active or inactive) in the cellular debris was not conducted.

Inhibition of enolase activity by small molecules was excluded as a possibility for the apparent loss of enolase activity by dialyzing crude extracts from various stages of sporulation against 50 mM Tris-5 mM MgCl_2 -10 mM 2-mercaptoethanol. The specific activity was the same after dialysis as it was before dialysis. Inhibition of enolase by nondialyzable, large molecules was ruled out based on mixing experiments. These experiments clearly showed that there were no large molecules capable of either inhibiting or activating enolase activity in crude extracts of sporulating cells.

Evidence that enolase is degraded (probably by the same mechanism involved with general protein turnover) arises from studies involving quantitation of enolase protein during sporulation using immunoprecipitation techniques.

These experiments show that the amount of enolase protein corresponds to the level of enolase activity in the cell (Table 5). An assumption in this type of experiment is that the antibody will recognize inactive or partially degraded enolase protein. It is worthwhile to note that there is no temporal separation between the loss of enolase activity and enolase protein. This suggests that the initial events of degradation are the rate limiting steps in proteolysis since no inactive precursor detectable with antibody accumulates. This same type of situation is also found with ATCase inactivation and degradation (36).

Further evidence will now be presented in an attempt to link enolase degradation with general protein turnover. Data indicate that the enzyme(s) involved in enolase degradation are synthesized following exponential growth. The amount of enolase activity does not vary with time in cultures which have been treated with chloramphenicol. The addition of chloramphenicol at T_4 has no effect on the normal pattern of enolase degradation.

Enolase activity begins to decrease at about T_3 , coincident with the initiation of protein turnover. The rate of enolase degradation is equal to the rate

of protein turnover. The similarities between the initiation and rate of enolase degradation and protein turnover suggests a link between the two events. The correlation with protein turnover has not been observed in the ATCase and amidotransferase systems where inactivation and degradation could be separated (temporally and metabolically) from protein turnover (36).

Enolase degradation and protein turnover both require the development of a proteolytic system that requires the continuous generation of metabolic energy and which is blocked by the early addition of chloramphenicol. The addition of 2,4-dinitrophenol to a culture at T_4 blocks the decrease in enolase activity.

The question of the mechanism of enzyme inactivation is a difficult one to approach. It would appear that enolase is not inactivated in the same fashion as ATCase or amidotransferase. In these systems, there is a rapid loss of activity that cannot be attributed to the dilution of activity by growth or general protein turnover. Enzymological, immunological, and kinetic data do not indicate whether or not enolase is inactivated immediately prior to degradation.

Enzyme inactivation is often thought to be a means of regulation of metabolic pathways. Because of the important metabolic position of enolase during sporulation, total inactivation of the enzyme would block gluconeogenesis and effectively block formation of spore associated polymers. On the other hand, a combination of synthesis and degradation could ultimately yield the precise level (or optimal level) of enzyme necessary for its function and ultimate incorporation into the developing spore.

The Relationship of Enolase Degradation to Sporulation

It is often difficult to establish whether a particular biochemical event in a Bacillus is sporulation specific or sporulation associated.

Asporogenous mutants blocked at various stages of sporulation are useful in determining which post-exponential changes in cell properties are sporulation specific (14). Unfortunately, such mutants are not available in B. licheniformis, therefore making proper analysis of sporulation specific events difficult.

An alternative approach is the use of various chemical agents to block sporulation. m-Amidobenzeneboronic acid (ABBA) blocks sporulation but does not interfere with vegetative growth (18). In an attempt to determine the relationship between enolase

degradation and sporulation, a culture of B. licheniformis was treated with ABBA. This culture did not sporulate, but the decrease in enolase activity was normal.

In B. subtilis, ABBA represses the synthesis of an intracellular protease(s) activity (18). Presumably, this is also true in the closely related organism B. licheniformis, and thus, at first consideration, it would appear that intracellular protease(s) is not involved in the post exponential loss of enolase activity. However, it is not known how effectively ABBA blocks intracellular protease synthesis nor just how much protease activity is required for the post-exponential but non-sporulating cell to inactivate enolase.

Evidence That the Loss of Enolase Activity is Not Due To the In Vitro Instability Of the Enzyme

Often times the inability to detect an enzymatic activity in crude extracts is due to the in vitro instability of the enzyme. The enzyme fructose - 1,6-biphosphatase (38) and pyruvate kinase (61) from B. licheniformis are two such examples in which the lack of a stabilizing ligand in the extraction buffer destabilized the enzyme and resulted in inaccurate data.

The sensitivity of some enzymes to proteolysis provides an example in which the in vitro activity of an enzyme was altered. Inosine-5-monophosphate dehydrogease (IMPDH) activity was reported by Deutscher and Kornberg (12) to disappear during sporulation. The first reports suggested that the IMPDH was inactivated similarly to ATCase. More recent studies have shown that the loss of IMPDH activity was an artifact due to in vitro proteolysis and that by washing the cells with 1 M KCl and including PMSF in the extracts, the loss of activity could be prevented (34).

Enolase activity in crude extracts was stable throughout a 5 h period, regardless of the stage of growth or sporulation from which the extracts were prepared. The stability of enolase was also independent on the type of growth medium (minimal vs. complex). The results suggest that enolase is stable in crude extracts prepared in 50 mM Tris-5 mM $MgCl_2$ -10 mM 2 ME, pH 7.6.

A further extension of these results is that the washing procedure effectively removed extracellular proteases and the PMSF completely inhibited any

intracellular protease activity. The fact that enolase activity was stable in extracts even in the absence of PMSF (data not shown) might also suggest that enolase is intrinsically resistant to proteolytic degradation. It is probable that the continued generation of metabolic energy is required for enolase degradation and that this was absent in a cell free extract.

Synthesis of Enolase

Antibody produced in rabbits against pure enolase precipitates ^{14}C -leucine labeled material from a crude extract with a high degree of specificity. Following dissociation and SDS gel electrophoresis, all of the radioactivity was associated with a single band that had a mobility identical with that of authentic enolase subunits. Furthermore, enolase activity could not be measured in extracts treated with an excess of this antiserum.

This highly specific antibody was used to study the synthesis of enolase during sporulation. The rate of enolase synthesis was highest during exponential growth and early sporulation and accounted for approximately 1-5% of the label incorporated during the 20 min labeling period. At T_2 , a dramatic decrease in apparent

enolase synthesis was detected. The rate of incorporation into the enzyme decreased approximately 8-fold during T_2 and T_3 , falling to about 0.2% of the total label incorporated in 20 min. This decrease could have been the result of either a genuine decrease in the rate of synthesis or an increased rate of degradation of enolase. It was possible to rule out an increased rate of degradation of enolase for the following reason. If the rate of enolase synthesis remained constant throughout sporulation, an increased rate of degradation would be necessary to account for the decrease in the rate of incorporation of radioactive label into enolase. To effect this kind of change, the half-life of the enolase protein would have to have been less than 20 min. A parallel decrease in enolase activity should have been, but was not, detected. It is improbable that newly synthesized enolase was more susceptible to proteolysis than pre-existing enzyme. Thus, it seems unlikely that an increase in enolase degradation is causing the decrease in the rate of incorporation of label into enolase.

The alternative explanation, that a decrease in the rate of enolase synthesis was responsible for the lower rate of incorporation of labeled into

enolase, seems more plausible. The mechanism of achieving this reduced rate of synthesis is unknown but could be explained by invoking a model involving selective transcription and/or compartmentalization of the enzyme.

The changes in the cell's metabolism, enzyme complement, and macromolecular composition during sporulation suggest that selective, ordered gene transcription must play an important role in the control of sporulation. Competition hybridization studies have shown that sporulating cells transcribe "vegetative" genes in addition to "sporulation" genes (58). The synthesis of sporulation associated products is easy to detect and quantify, but the continued synthesis of vegetative gene products is difficult to monitor since it requires the means to specifically isolate those proteins. ATCase has been used as an example of a purely vegetative gene product, that is, a protein required only during growth. It has been demonstrated that ATCase synthesis ceases upon the cessation of exponential growth (35). In this study, it has been demonstrated that enolase, a protein required at all stages of growth and sporulation, is synthesized throughout sporulation. Although the rate of synthesis is significantly lower than the

rate observed during exponential growth, it is, none-the-less, detectable. Thus, some sort of selective modulation of enolase transcription must occur.

One possible mechanism of selective transcription is at the level of the enzyme RNA polymerase. Several investigators have looked for a modified form(s) of RNA polymerase which preferentially transcribes sporulation associated genes. Most recently, the discovery of several new sigma like factors, termed delta factors, has reinforced the possibility of RNA polymerase modification during sporulation. These peptide factors, described in B. subtilis, become associated with the core RNA polymerase at about T_3 (16). It is about this time that the rate of enolase synthesis changes. Differences in the cation requirements and template specificity of the RNA polymerase also occur at this time.

It is possible that the enolase gene cannot be transcribed as efficiently by the sporulation form of the polymerase as by its vegetative form. Yet some of the enolase is still transcribed.

Another possibility to consider is the sequestering of enolase in the forespore which is separated from the mother cell by two membranes. Enolase would be required for germination and outgrowth and the inclusion

of at least a minimal amount of enolase in the spore is requisite. Since the amount of enolase is decreasing in the entire cell during sporulation, it is logical that newly synthesized enolase might be deposited where it is needed, i.e., in the forespore compartment. Enolase could either be synthesized completely within the forespore or perhaps on membrane bound ribosomes and transported into the forespore.

It is possible that selective translation could also be involved in enolase metabolism during sporulation. However, convincing evidence of a selective translation process in sporulating cells has not been reported.

It would be possible to test the validity of the compartmentalization model if the forespores were separated from the mother cell compartment. Using the same pulse labeling technique utilized in this investigation, enolase could be labeled, the compartments separated and the location of the labeled enolase determined following precipitation with antibody. Such experiments would be better conducted in the B. megaterium system where the technique for the separation of the compartments has been perfected (53).

SUMMARY

Enolase has been purified from B. licheniformis. The enzyme is very similar to the enolase from B. megaterium in physical and kinetic properties and is immunologically related. It appears that the enolases from the genus Bacillus are more closely related to the enolases isolated from the genus Thermus than to those from E. coli or yeast.

B. licheniformis does not sporulate efficiently in a glucose-NH₄⁺-salts minimal medium. Enolase activity remains constant until at least T₈ and represents a balance between synthesis and degradation of the enzyme. A problem with reutilization of labeled amino acids even in the presence of a large excess of unlabeled amino acid was incurred during sporulation. This reutilization made it impossible to use conventional pulse-chase technique for determining turnover rates. It was interesting to note that a loss of label from general protein was detectable and increased to 13% hr⁻¹ during sporulation.

In contrast to minimal media, B. licheniformis sporulated well in SNB media. Enolase activity could

be identified in crude extracts prepared from all stages of growth and sporulation and reached a maximum value at T_2 . Subsequently, the enzyme activity and protein decreased at a rate of $10\% \text{ hr}^{-1}$. The decrease in enolase activity and protein appear to be related to the initiation of protein turnover. The decrease of enolase activity is sensitive to the early addition of chloramphenicol and the addition of 2,4-dinitrophenol. Inhibition of protein synthesis and energy metabolism also inhibits general protein turnover.

Enolase synthesis could be detected throughout growth and sporulation. The highest rate of synthesis was detected during exponential growth and early sporulation. At T_2 , the rate of enolase synthesis decreased 8-fold. A speculative model is presented in which the regulation of the concentration of enolase in sporulating cells is achieved by selective transcription and compartmentalization.

This study has demonstrated that a protein required during vegetative growth, sporulation, and germination is not immune to degradation during sporulation. While purely vegetative enzymes are inactivated and their synthesis repressed, enolase, which must participate in all stages of growth,

continues to be synthesized at the same time it is degraded during sporulation. Thus, to generalize, all proteins in a sporulating cell are subject to turnover and the amount of an enzyme present in the completed spore represents a balance between enzyme synthesis and degradation.

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