FURTHER CHARACTERIZATION OF THE PURINE NUCLEOSIDE PHOSPHORYLASES OF BACILLUS CEREUS SPORES AND VEGETATIVE CELLS

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ABSTRACT

FURTHER CHARACTERIZATION OF THE PURINE NUCLEOSIDE PHOSPHORYLASES OF *BACILLUS CEREUS* SPORES AND VEGETATIVE CELLS

By

Helen Louise Engelbrecht

The purine nucleoside phosphorylases of *Bacillus cereus* spores and vegetative cells were each purified to a state of electrophoretic homogeniety. The enzymes had previously been shown to be the products of one cistron and were similar in many properties. They were identical in their pH-activity spectra with optima at 8.3 and 7.7 depending on the method of assay. The molecular weights of the enzymes in the presence of excess phosphate were approximately 110,000. The Michaelis constants for the spore and vegetative cell purine nucleoside phosphorylases (PNPase) for phosphate were 7.3×10^{-3}M and 5.1×10^{-3}M respectively. Both PNPases were affected by sulfhydryl reagents and their activities were enhanced to differing degrees by manganese. The turnover numbers for the spore and vegetative cell enzymes were calculated to be 128 and 186 moles of inosine per mole of enzyme per second, respectively. The PNPase from vegetative cells was more anionic than that from the spores during gel electrophoresis in low concentrations of phosphate buffer. The

Stokes' radii and sedimentation constants of the vegetative cell enzyme were constant over a wide range of phosphate concentrations. However, these parameters of the spore enzyme were concentration dependent with respect to phosphate ion. The spore and vegetative cell enzymes were identical at phosphate concentrations above the Michaelis constants for phosphate. As a consequence, the molecular weight of the spore enzyme increased from approximately 89,000 to 117,000 while that of the vegetative cell enzyme remained unchanged at 110,000 as the phosphate concentration was increased from zero to 0.05M. The half-life of spore PNPase at 50C was approximately 75 minutes in the absence of phosphate, which decreased to 10 minutes in 0.05M phosphate. The heat resistance of the spore enzyme in phosphate was equal to that of the vegetative cell PNPase.

The data support the hypothesis that the vegetative PNPase undergoes modification during sporulation to yield an active enzyme whose state of aggregation and other physical properties varies with the ionic environment.

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By

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INTRODUCTION

Members of the Bacillaceae are Gram positive bacteria. They are capable of forming endospores which are usually heat resistant and metabolically dormant. The process of sporulation is of interest because it is a well-documented case of morphogenesis which can be viewed superficially as a case of abortive cell division. The spore structure is quite complex and differs from that of the cell. Spores are composed of an inner core (protoplast) which contains ribosomes, nucleic acids, and enzymes, which are bounded by a typical membrane. Outside the membrane is a cortical layer of peptidioglycan composition. It appears electron transparent by most commonly used fixation techniques for electron microscopy. The cortex is surrounded by numerous proteinaceous lamellar layers composing the spore coat. Many spores also have an exosporium composed of protein and lipid which appears as a loose fitting coat. While the spore is able to remain in a dormant state for long periods of time, it is also capable of rapid germination and outgrowth to form vegetative cells under proper external conditions.

Many of the proteins found in the spores are similar catalytically, to those of vegetative cells. Such is the case for the spore and vegetative cell purine nucleoside phosphorylases of *Bacillus cereus*. The synthesis of these enzymes was shown to be controlled by a single genomic unit (19). Therefore, it would be expected that the proteins in the two forms would be identical. However, the enzymes have strikingly different properties in the intact cells or spores. Unlike the vegetative cell enzyme, the spore phosphorylase is heat stable and dormant *in vivo*. These differences in properties may be the result of the unique "packaging" in the spore or may reflect some structural modifications of the enzyme which occurs during sporulation. A partial understanding of the basis of the differences in protein properties could be obtained by purifying the enzymes and studying them under identical conditions.

Enzymes are the most readily studied proteins of spores because of their catalytic activity. The purine necleoside phosphorylases are present in spores and vegetative cells in quantities great enough to permit extensive purification and they are stable during ammonium sulfate fractionation, gel filtration, and preparative gel electrophoresis. Gardner and Kronberg (19) reported that the highly purified spore enzyme differed in subtle ways from the vegetative cell enzyme in pH response,

sedimentation velocity, and substrate specificity. Therefore, it was decided that the enzymes merited further characterization to determine if they were in fact identical or similar enzymes.

The purpose of this thesis was, therefore, to characterize the purine nucleoside phosphorylases from spores and vegetative cells and to determine if the enzymes were identical or only similar. The enzymes were purified to electrophoretic homogeniety and their physical chemical and functional properties were studied.

LITERATURE REVIEW

Numerous and extensive review articles have appeared in recent years concerning various aspects of sporulation and germination in the Bacillaceae (26, 46, 60, 82, 86). These publications are evidence for the widespread interest and efforts being expended in understanding this system of unicellular morphogenesis. The process may be visualized as an abortive cell division in which one product subsequently develops into a resting cell form. Little is understood of the various biochemical changes which occur during sporulation.

In the exponential growth of cells, there is a depletion of carbohydrates and a building up of metabolic products, principally acetate in *Bacillus cereus* (24, 27, 62). There is a shift from an almost completely Embden-Meyerhoff metabolism to the tricarboxylic acid and diacetyl oxidation cycles (43). At this time there appears a synthesis, in a temporal sequence, of sporulation-specific enzymes, particularly protease (7). This enzyme is thought to promote protein turnover in that part of the sporulating cell which becomes the sporangium. During the course of protein turnover, there is loss of some catelytic properties and the

loss of solubility of the electron transport system (14). There is an apparent cessation of DNA synthesis, but early in sporulation, new macromolecular synthesis involves lipid synthesis as part of membranes to encase DNA. Later, the carbohydrate and protein structural materials are synthesized. The last acts of the sporulation process are the synthesis of small spore-specific molecules such as dipicolinic acid which accounts for up to 15% of the dry weight of the spore. At this time, the uptake of divalent cations such as manganese and calcium also occurs.

Purine nucleoside phosphorylase (PNPase) is normally repressed in exponentially growing vegetative cells, but is derepressed at the time of sporulation. Alternatively, PNPase can be induced in cells by addition of inosine (19). It appears at approximately the same time as glucose dehydrogenase (2), an "early" enzyme in the sporulation process. The role of PNPase in the sporulation process is not understood.

Purine Nucleoside Phosphorylases

Nucleoside phosphorylases have been studies because of their ability to produce nucleosides and were thought to be in the pathway of nucleic acid synthesis. A number of the early studies were prompted by the discovery that crude hemolysates of animal tissue, particularly red

blood cells were able to degrade such compounds as adenosine (13, 42).

Purine nucleoside phosphorylases (PNPase) were found in human erythrocytes (88), rat liver (33), *Escherichia coli* (56), yeast (30), and the Bacillaceae, particularly *B. cereus* (48, 49, 50).

Kalckar (33, 34, 35, 36) isolated the first nucleoside phosphorylase from rat liver. He showed that equimolar ribose-1-phosphate and hypoxanthine produced equimolar inosine and phosphate. The equilibrium of the reaction was shown to favor inosine with 70-80% conversion. The reaction was driven to completion if the inorganic phosphate was removed. The rat liver enzyme was specific for the production of inosine and guanosine.

Manson (56) found that the purine nucleoside phosphorylase enzyme of *Escherichia coli* was able to cleave guanosine and inosine. The enzyme in yeasts was able to cleave inosine, guanosine, and nicotinamide riboside (30).

In the case of the Bacillaceae, the interest in PNPase was primarily the result of finding that adenosine was important in the initiation of germination and that the cleavage of purine ribosides did in fact occur in the spore. Lawrence (48, 49) first described the cleavage of adenosine and the recovery of adenine and free ribose by *Bacillus cereus*. Ribosidase activity, bound to the spore debris, was directed toward adenosine and inosine according to the observations of Powell and Hunter (70). Krask and Fulk (47) found that extracts of *B. cereus T.* contained nucleoside phosphorylase which cleaved adenosine or inosine yielding ribose-1-phosphate. They also presented evidence that a deaminase acted on adenosine prior to phosphorolysis.

Purine nucleoside phosphorylase of *B. cereus* was highly purified and characterized by Gardner and Kornberg (19). These investigators achieved specific activities of 3,790 and 4,590 μmoles inosine cleaved per hour per milligram protein for the spore and vegetative cell enzymes, respectively. They concluded that the enzyme isolated from spores (sPNPase) and vegetative cells (vPNPase) was the same in those physical and catalytic properties which they studied. The enzyme was capable of using deoxyinosine, deoxyguanosine, and 6-mercaptopurine riboside as well as inosine and guanosine as substrates with varying degrees of efficiency. The molecular weight was estimated to be 80,000 based on their observed sedimentation constant of 4.8s. The enzyme had a Km of 1.1-1.4 x 10^{-4} with respect to inosine. There were some quantitative differences in pH response of the vegetative cell and spore enzymes and differences with respect to their abilities to use guanosine as substrate.

Spore Enzyme Heat Resistance

In order to achieve heat resistance, an *in vivo* stability is required of all spore enzymes essential for the viability of the spore. Most enzymes from spores are stable *in vivo* but they are labile *in vitro*. Thus it appears superficially that spores are stable because of the "packaging" of their labile components. A number of partially purified spore enzymes have been shown to be intrinsically stable or capable of stabilization by environmental factors. The NADH oxidase of *Clostridium botulinum* (22) and the catalase of *B. cereus* (76) appeared to be stable on extraction from spores. The heat resistance of glucose dehydrogenase can be varied over a million-fold range depending on the conditions of pH and ionic strength (78). At pH 6.5 and in 3M solutions of sodium chloride, the enzyme is as stable *in vitro* as it is in the spore. Those conditions which produced the greatest heat resistance also brought about disaggregation of the molecules. The heat resistance of fructose-1, 6-di-phosphate aldolase of spores of *B. cereus* was enhanced 10-15 fold by 10^{-2}M solutions of calcium chloride (80). It is significant that the calcium ion concentration in spores is 1.5M, assuming equal calcium and water distribution throughout the spore volume.

These kinds of observations suggested that heat resistance of proteins in spores may be related to their

structure and the heat resistance of spores was achieved through these molecular structures fitting into a unique cellular structure or environment.

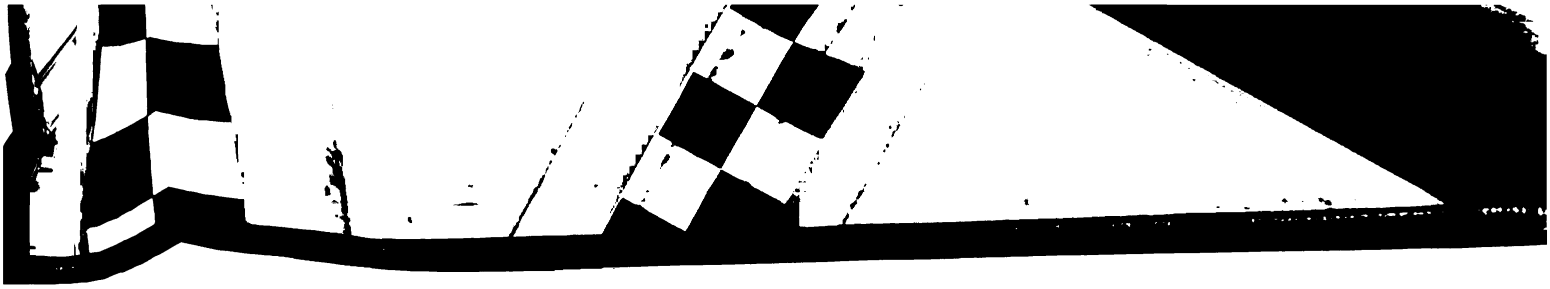
MATERIALS AND METHODS

Cells, Spores, and Their Extracts

Bacillus cereus is a member of the Bacillaceae and forms endospores upon completion of exponential growth. The organism employed in this research was originally isolated at the University of Illinois and has been variously known as *Bacillus terminalis* (84) and *Bacillus cereus T* (8). This strain produces an enzyme which lyses the sporangia so that free spores are easily obtained.

Preparation of Medium

Sporulation levels of 95% or greater were achieved in semi-synthetic growth medium, G medium (29, 84). The modified "G" medium contained the following materials per liter: K_2HPO_4, 1g; $(NH_4)_2SO_4$, 4g; $MnSO_4 \cdot H_2O$, 0.1g; $MgSO_4$, 0.8g; yeast extract, 2g; glucose, 4g; $ZnSO_4$, 0.01g; $CuSO_4 \cdot 5H_2O$, 0.01g; $CaCl_2$, 0.1g; and $FeSO_4 \cdot 7H_2O$, 0.001g. Dow Corning antifoam AF was also added at the rate of 1 milliliter per liter. The organism grew and sporulated well in either large or small quantities at 30C. For convenience in extraction and preparation of enzyme, one hundred liter batches of vegetative cells and spores were produced.



Preparation of Inoculum, Cell and Spore Crops

To obtain the levels of sporulation described, it was necessary to attain 3 liter volumes of exponentially growing cells. This was achieved by developing the following schedule of transfers. The stock culture was maintained in the sporulated state at 4C on nutrient agar.

A fresh nutrient agar slant was inoculated from stock and incubated for six to eight hours. Fifty milliliters of "G" medium in a 500 milliliter Erlenmeyer flask were inoculated from the slant and the flasks were shaken for two hours. Two similar flasks were inoculated with 5 ml from the first flask. After two hours, the 110 milliliters of culture were introduced into three liters of "G" medium in a New Brunswick fermenter (New Brunswick Scientific Co. New Brunswick, N. J.). An active inoculum for the 100 liter culture was attained after two hours of growth.

Log phase vegetative cells were prepared in the 100 liter stainless steel fermenter (Stainless Steel Products Co.) and harvested after five to six hours. The culture sporulated and lysed to produce free spores in approximately twenty hours. In both cases, the medium was cooled and the cells harvested in a Sharples centrifuge, Model A5-12. They were stored as a frozen paste at -15C. Approximately 500 grams wet weight of either spores or vegetative cells were obtained by this method.

Enzyme Extraction

Vegetative cells.--Purine nucleoside phosphorylase was extracted from vegetative cells of *Bacillus cereus* and it was purified by successive ammonium sulfate fractionations and gel filtrations. Five hundred grams of frozen cells were thawed and resuspended in 1200 milliliters of 0.05M Tris-HCl buffer pH 7.5. The suspension was placed in an Eppenbach colloid mill (Model MV-6-3 Gifford-Wood Co., Hudson, N. Y.) which was cooled by circulating alcohol at -20C. The initial rotor-stator distance was 0.06 inches (wide separation). Four milliliters of antifoam were added to the suspension and the mill was brought to about 25% of the full speed. Seven hundred and fifty grams of glass beads (No. 110 pavement marking beads, Minnesota Mining and Manufacturing Co.) were added slowly. The speed of the mill was increased to approximately 85% of the full speed and the rotor-stator gap was reduced to 0.03 inches. The cells were subjected to this treatment for ten to fifteen minutes and drained from the mill. Purine nucleoside phosphorylase was present in the supernatant fluid which was obtained on centrifugation of the extract.

Spores.--Purine nucleoside phosphorylase was extracted from spores of *Bacillus cereus* by an extraction procedure which was the same as that employed for vegetative cells

with the following exception: The spores were subjected to 85% of the full speed of the colloid mill for a minimum of thirty minutes. On the basis of equal wet weights of cells and spores, approximately twice as much enzyme was recovered from spores as from vegetative cells.

Enzyme Assay

The two methods (19) were used to assay for the presence of purine nucleoside phosphorylase. A colorimetric assay was based on the arsenolysis of inosine in the presence of enzyme and the measurement of the resulting ribose by a sensitive reducing sugar assay (67). It was carried out by the addition of the following meterials to 18 x 150 mm pyrex tubes.

0.1 ml of enzyme solution
0.2 ml 0.05M Tris-HCl pH 7.5
0.1 ml 0.05M Sodium arsenate
0.1 ml 0.005M Inosine

The reaction was started with inosine and incubated for 10 minutes in a waterbath at 25C. The blank contained

0.3 ml 0.05M Tris-HCl, pH 7.5
0.1 ml 0.05M Sodium arsenate
and 0.1 ml 0.005M Inosine.

The tubes were placed in a boiling water bath for two minutes to stop the enzyme reaction. One half milliliter of water was added to each tube to bring the volume to 1.0 ml. The sugar contents of the reactions mixtures, blank, as well as the standards containing 1-9µg of ribose were determined in the following manner:

To each tube was added:

1 ml carbonate-cyanide reagent (2.65 g $NaCO_3$
and 0.325 g KCN per 500 ml.)

and

1 ml potassium ferricyanide (0.25 g/500 ml)

The tubes were stoppered with glass spheres (marbles) and were heated in a boiling water bath or steamer for fifteen minutes. After cooling in ice, 5 milliliters of ferric iron reagent (0.75 g Ferric Ammonium Citrate and 0.5 g of Duponol were dissolved in 500 ml 0.05N H_2SO_4) were added. Fifteen minutes later, the samples were transferred to cuvettes and read against a reagent blank at 690 mµ. One unit of enzyme was defined as that amount which catelyzed the hydrolysis of 1 µmole of inosine per hour (19). Therefore, 1 µmole or 150 µg of ribose was produced per hour per unit of enzyme. The specific activity was µmoles of ribose per hour per milligram purine nucleoside phosphorylase (PNPase).

The spectrophotometric assay of PNPase at 290 mµ is dependent on the conversion of hypoxanthine to uric acid in the presence of excess xanthine oxidase (36). Hypoxanthine results from the cleavage of inosine by purine nucleoside phosphorylase.

A reaction mixture of one milliliter contained 0.6 ml of 0.05 M potassium phosphate buffer, pH 7.5, 0.2 ml

inosine, 0.01 M ml xanthine oxidase containing a minimum of 0.5 units and 0.1 ml purine nucleoside phosphorylase. One unit of xanthine oxidase was that amount forming one micromole of uric acid per minute at 25C. The increase of optical density at 290 mµ was measured in a Beckman DU spectrophotometer equipped with a Ledland log converter and a Sargent SR recorder. The sample compartment was maintained at 37C. The reaction was originally started by addition of inosine. However, the inosine was relatively unstable and spurious results were often obtained. Therefore, the reaction was initiated by addition of the enzyme so that background levels of hypoxanthine could be detected.

Xanthine Oxidase Preparation

A large number of spectrophotometric assays of PNPase were performed during the course of this research requiring a large quantity of xanthine oxidase. The enzyme was prepared from unpasteurized cow's milk according to the method of Gilbert and Bergel (20). Fifteen liters of raw milk were obtained from the Michigan State University dairy and run through a cream separator (Alfa Laval, Stockholm). Three liters of cream were obtained. One milliliter of 20% (w/v) sodium salicylate was added per liter and the mixture was placed in a New Brunswick fermenter without aeration. It was "churned" for three

hours at 4C to aggregate the fat globules in order to remove some of the butterfat.

The preparation was titrated to pH 7.5 with $NaHCO_3$ (17 g/l), cysteine-HCl (0.3 g/l) and EDTA (ethylenediamine tetraacetic acid) 0.37 g/l. The cream was digested with 1:250 trypsin (1.6 g/l) for 3.5 hours at 37C in a water-bath.

The mixture was cooled to -2 to 0C in an alcohol bath (-20C) with constant stirring, followed by treatment with 0.167 volumes of butan-1-ol (pre-cooled to -20C) and 190 grams $(NH_4)_2SO_4$ per liter. This preparation was stirred for one half hour then allowed to stand 16 hours at -4 to 4C. The clear aqueous middle layer was drawn off and saved. The aqueous portion of the top layer was obtained by centrifugation and pooled with the aqueous middle layer. The precipitate and butan-1-ol fractions were discarded.

The aqueous layer and 110 grams $(NH_4)_2SO_4$ per liter were stirred thirty minutes and allowed to stand 1-2 hours at -4 to 4C. The precipitate which formed was collected by centrifugation. It was resuspended and dialyzed against 0.2M potassium phosphate buffer pH 6.0 containing 0.2 milligrams sodium salycilate per milliliter. This preparation was then brought to 60% weight/volume with $(NH_4)_2SO_4$ and stored in a brown bottle at 4C.

Xanthine Oxidase Assay

Xanthine oxidase decayed in storage and was assayed daily to insure adequate activity in the reaction mixtures. The assay was performed at 25C in three milliliter cuvettes containing

1 ml 0.1M potassium phosphate buffer, pH 7.5
1 ml deionized distilled water
0.1 ml hypoxanthine (10 milligrams/500 ml)
0.1 ml xanthine oxidase

and read at 290 mμ in the Beckman DU Spectrophotometer described previously. One unit of xanthine oxidase was that amount forming one micromole urate per minute at 25C. A change in optical density of 0.06 per 0.1 ml or 0.05 units of xanthine oxidase was sufficient to perform the purine nucleoside phosphorylase assays.

Estimation of Protein

Estimation of protein was performed spectrophotometrically by the method of Warburg and Christian (90) or by the method of Lowry (53). Protein concentrations were reported as milligrams of protein per milliliter of preparation tested.

Acrylamide Gel Electrophoresis

Preparative Gel Electrophoresis

Jovin *et al*. (32) developed an apparatus for preparative temperature-regulated polyacrylamide electrophoresis

in discontinuous buffer systems. The apparatus utilized the resolving power of the system previously developed by Ornstein and Davis (66) with various modifications in design. The Canalco preparative gel electrophoresis unit (Canalco Instrument Co. Rockville, Maryland) was derived from these two designs.

The gel system used for enzyme purification reported in this thesis consists of a spacer gel of 8 milliliters and a separation gel of 16 ml volume. The spacer gel was used to resolve the proteins according to their charge and size and permit them to equilibrate and separate into definite bands. The pH of this gel was approximately 6.5. The separation gel was a more highly cross-linked gel which caused the molecules to separate more on the basis of size. This permitted molecules of similar unit charge to be separated with respect to their sizes. Elution from the column was accomplished by a flow of 1 ml of buffer per minute across the lower face of the gel column. The eluate was collected in five minute fractions. The gel was prepared in the following manner. Stock solutions of various components of the system were maintained.

<u>Solution A</u>

240 ml 1N hydrochloric acid
1.15 ml N,N,N'N'-Tetramethylethylenediamine(TEMED)
181.5 gms. trihydroxyamino methane (Tris)
Distilled water to 500 ml pH 8.8-9.0

Solution CN

40 g acrylamide

0.12 gms. N,N'-Methylenebisacrylamide (Bis)

Distilled water to 100 ml.

Solution B

48 ml 1N hydrochloric acid

5.98 gms. Tris

0.46 ml TEMED

Distilled water to 100 ml pH 6.6-6.8

Solution DN

14 gms. acrylamide

0.25 gms. Bis

Distilled water to 100 ml.

Solution E

4 milligrams Riboflavin

Distilled water to 100 ml.

The separation gel containing

1 part A

2 parts CN

1 part H_2O

4 parts catalyst containing ammonium persulfate, 0.2 g per 100 ml water

was layered into the column and covered with 0.5 ml water. After two hours, a definite line was seen between the gel and water indicating that polymerization had occurred. The water layer was withdrawn and a spacer gel containing the following was added:

1 part B

2 parts DN

1 part E

4 parts distilled water.

Water, 0.5 ml was layered on the top and the gel column was exposed to fluorescent lights for approximately two hours to effect polymerization.

The electrode buffer was tris-glycine buffer, pH 8.3 containing 3 gms Tris and 14.4 gms glycine per liter distilled water.

The elution buffer contained solution A diluted 1 part per 7 parts distilled water.

Partially purified purine nucleoside phosphorylase, two to twelve milliliters containing less than 25 milligrams of protein from spores or vegetative cells was applied to the column. The enzyme was made to 8% w/v with sucrose and 0.2 ml of 0.01% bromphenol blue was added as an anionic marker.

Sufficient voltage was applied to cause a current of 5 milliamperes (ma) to flow. When the enzyme had moved into the spacer gel, the current was raised to 15 ma and maintained at that level throughout the run. A peristaltic pump was installed between the buffer reservoir and the column to move the elution buffer over the face of the column (Canalco Technical Bull.). Cold water was also pumped through the cooling jackets of the apparatus. The

elution was carried out throughout the run, but fractions were not collected until the anionic marker started to elute from the column. The enzyme was eluted from the column fifteen to thirty tubes after the bromphenol blue band.

Analytical Gel Electrophoresis

The upper limit for protein resolution in the analytical disc electrophoresis system (66) was 150 μg. The composition of the analytical gels and buffers was similar to the preparative gel. However, more Bis was included to give a greater extent of cross-linkage than was used in the preparative gels.

The stock solutions for these gels were as follows:

Solution A pH 8.9

48 ml 1N Hydrochloric acid
36.6 gms Tris
0.23 ml TEMED
Distilled water to 100 ml.

Solution B pH 6.7

2.8 ml 1N HCl
5.98 gms Tris
0.45 ml TEMED
Distilled water to 100 ml.

Solution C

20.0 gms Acrylamide

0.735 gms Bis

Distilled water to 100 ml

Solution D

10.0 gms Acrylamide

2.5 gms Bis

Distilled water to 100 ml

Solution E

4 mgms Riboflavin per 100 ml distilled water

Solution F

40 gms Sucrose per 100 ml distilled water

Catalyst

0.14 gms Ammonium persulfate per 100 ml distilled water

Small pore or separation gel

1 part A

2 parts C

1 part distilled water

4 parts catalyst

Large pore or spacer gel

1 part B

2 parts D

1 part E

4 parts F

The analytical gels were polymerized in glass tubing 7.5 cm length with an internal diameter of 0.5 cm. The separation gel was poured to a height of 4 cm, layered with

water, and allowed to polymerize for about forth-five minutes or until a definite line was observed at the gel-water interface. The water was withdrawn and 3 mm of large pore spacer gel was added to the column, again overlaying with water. The gels were exposed to fluorescent light for 5-10 minutes or until the gel formed a cloudy appearance. At this time, the gels were moved to the electrophoresis apparatus (Buchler Instruments Co.) and allowed to equilibrate at 4C prior to adding the samples. The gels were run in duplicate at 2.5 ma per tube using bromphenol blue as a marker.

The standard electrode buffer was Tris-glycine buffer, pH 8.3. The stock buffer contained 28.8 gms glycine and 6 gms Tris per liter of distilled water. This solution was diluted 1:10 for use. Potassium phosphate buffer, pH 8.3 was added at varying molarities for studying the phosphate effects.

One gel of each duplicate set was stained with 1% Amido Schwarz in 7% acetic acid (12) and subsequently destained by electrophoresis in 7% acetic acid. The duplicate was stained for purine nucleoside phosphorylase according to a modification of the method of Mattson and Jensen (58) by which reducing sugars are determined using triphenyl tetrazolium chloride. This method causes the dye marker to disappear. Therefore, it was necessary to

measure the length of the column and the distance traveled by the dye marker prior to staining for the enzyme.

The modified procedure was as follows:

0.3 ml 0.05M Tris-HCl buffer pH 7.5,

0.1 ml 0.01M Inosine, and

0.1 ml 0.05M Sodium arsenate were added to a 13 x 100 mm

test tube containing the gel. The gel was incubated for 15 minutes at 37C then washed thoroughly in distilled water. Five milliliters of 1N NaOH and 1 ml 0.5% triphenyltetrazolium chloride were added to the gel and placed in a boiling water bath for a few minutes until the red precipitate of formazan generated by the reduction of the tetrazolium formed in the gel. The gel was immediately removed, washed in cold water, and fixed in 7% acetic acid. Measurements of the length of the gel and the distance traveled by the enzyme were made.

Sedimentation

Sucrose density centrifugation was used to observe the sedimentation behavior of spore and vegetative cell purine nucleoside phosphorylases (57). The procedure was carried out in a SW 39 rotor containing plastic tubes 1/2" diameter by 2" length. The sample was layered at the top of a sucrose gradient.

The technique relies on the observation that the ratio of the distance from the meniscus traveled by a

given protein to the distance traveled by a standard of known $S_{20,w}$ is a constant. Therefore, the constant equals the ratio of $S_{20,w}$ unknown to $S_{20,w}$ standard. Thus one may determine the $S_{20,w}$ of the unknown with respect to the standard.

Linear gradients of cold 5-20% sucrose in

0.05M Tris-HCl pH 7.5

0.001M Potassium phosphate and 0.05M Tris-HCl
pH 7.5

0.01M Potassium phosphate and 0.05M Tris-HCl
pH 7.5

and 0.05M Potassium phosphate and 0.05M Tris-HCl
ph 7.5

were made using a double-chamber lucite block with a stirring motor. The gradients were kept under refrigeration until they were used. Human hemoglobin whose $S_{20,w}$ value was 4.2 was used as a standard. The sample contained 2.75 OD units of hemoglobin in 0.05 ml saline and 0.05 ml enzyme. They were layered on the gradients immediately prior to centrifugation at 38,000 rpm for 23.5 hours at 7C. A swinging bucket rotor SW-39 designed for the model L-Spinco centrifuge was used (Beckman Instruments Inc., Spinco Division, Palo Alto, California).

The samples were collected in three drop fractions from the bottom of the tubes by puncturing them with a double pronged needle. An average of 110 drops was collected per tube. Each fraction was assayed for the

presence of purine nucleoside phosphorylase. Hemoglobin was estimated spectrophotometrically at 450 mμ.

Gel Filtration

Ackers (1) demonstrated that the primary molecular sieving effect in Sephadex G-200 was a steric and frictional interaction of the solute molecule with the gel matrix. He developed a method of calibration involving the effective gel pore radius as a parameter. The columns could be calibrated with materials of known Stokes' radius and the size of an unknown macromolecule could be calculated on the basis of its effluent peak position.

The basic equation describing the mechanism of operation of a molecular sieve column is shown in Equation 1.

$$V_e = V_o + K_d V_i \text{ or } K_d = \frac{V_e - V_o}{V_i} \qquad (1)$$

where K_d = partition coefficient,

V_e = effluent volume,

V_o = void volume, and

V_i = internal volume of gel.

The Stokes' radius, a, of a macromolecule diffusing within a restrictive barrier of pore radius r is related by the Renkin equation to the equivalent free cross-sectional pore area A (Equation 2).

$$\frac{A_r}{A_o} = \left(1 - \frac{a}{r}\right)^2 \left[1-2.104\,\frac{a}{r} + 2.09\left(\frac{a}{r}\right)^3 - 0.95\left(\frac{a}{r}\right)^5\right] \quad (2)$$

where A_r = effective actual area and

A_o = equivalent cross-sectional area.

The equation has been extended to show approximately the diffusion restriction encountered by several macromolecules during migration in a molecular sieve chromatographic column. The effective solute distribution ratio is governed by steric and frictional hinderance and the distribution constant (K_d) is equal to the ratio $\frac{Ar}{Ao}$. Therefore equations 1 and 2 were combined (1).

$$\frac{Ve-Vo}{Vi} = \left(1 - \frac{a}{r}\right)^2 \left[1-2.104\,\frac{a}{r} + 2.09\left(\frac{a}{R}\right)^3 - 0.95\left(\frac{a}{R}\right)^5\right] \quad (3)$$

Ackers has solved equation (3) for $\frac{a}{r}$ values corresponding to a range of K_d values from 0 to 1. His solutions were used in assessing the Stokes' radius when the pore size of the column was known.

A G-200 column, 18 cm in height and 1.5 cm diameter was calibrated to determine the pore radius for the column. Blue Sephadex was used for finding the void volume. Human hemoglobin (Stokes' radius a = 3.08) was used as the known protein. Radioactive phosphate ($^{32}PO_4^{=}$) was used to determine the internal pore volume. In 0.05M Tris-HCl, pH 7.5, the pore radius was calculated to be 18.5 mμ.

The Stokes' radius of the vegetative cell and spore purine nucleoside phosphorylases were tested in the following buffers:

0.05M Tris-HCl pH 7.5
0.001M potassium phosphate and 0.05M Tris-HCl pH 7.5
0.01M potassium phosphate and 0.05M Tris-HCl pH 7.5
0.05M potassium phosphate and 0.05M Tris-HCl pH 7.5

to determine the effect of phosphate on the molecular size of the enzymes.

Molecular Weight Determinations

From the Stokes' radius and the sedimentation constants, it is possible to determine the molecular weight of a globular protein. Using the relationship:

$$MW = \frac{RTs}{D(1-\bar{v}\rho)} \quad (4) \quad \text{and}$$

$$D = \frac{RT}{Nf} = \frac{RT}{N6\pi a\eta} \quad (5)$$

$$\text{then } MW = \frac{6\ \pi\ \eta\ Nas}{(1-\bar{v}\rho)} \quad (6)$$

where R is the universal gas constant, T is the absolute temperature, s is the sedimentation constant, D is the diffusion constant, and $\bar{v}$ is the partial specific volume of the protein which in this research was assumed to be 0.725, N is Avagadros' number, a is the Stokes' radius, and ρ is the density of the suspending medium, and η the viscosity of the suspending medium. These relationships

were used to estimate the molecular weights of the enzymes at various levels of phosphate.

Purification and Assay of Protease

Protease from Bacillus cereus was prepared for use in testing for the conversion of the vegetative cell enzyme to the spore form by limited proteolysis.

The protease was obtained from supernatant media of a nineteen hour sporulating culture by the addition of $(NH_4)_2SO_4$ to 80% saturation and resuspension of the resulting precipitate in distilled water. This preparation was then dialyzed five hours against distilled water, changing the water every hour. The sample was combined with a dextran tracer and placed on a G-25 Sephadex column. The protease eluted in the void volume and was used directly off the column. One unit of protease was that amount which produced an OD of 1.0 at 340 mμ using 0.5 mg/ml azoalbumin as substrate.

The protease assay was dependent on the formation of trichloroacetic acid soluble chromophoric peptides from azoalbumin. The activity was measured as follows:

1 ml azoalbumin (5 mg/ml) and
0.2 ml protease solution

were incubated for 20 minutes at room temperature in 13 x 100 mm tubes. A blank contained:

1 ml azoalbumin
0.2 ml distilled water.

Two milliliters of 10% trichloroacetic acid were then added and the precipitates were removed by centrifugation in a clinical centrifuge. The absorbance of the supernatant solutions were read against the blank at 340 mμ.

EXPERIMENTAL RESULTS

Purification

Gel Filtration

In order to determine the characteristics of the purine nucleoside phosphorylases without interference of other cell components, it was necessary to obtain the cell and spore enzymes in a high degree of purity. Vegetative cell purine nucleoside phosphorylase (vPNPase) was prepared by successive treatments of extracts with streptomycin sulfate, ammonium sulfate fractionation, and acrylamide gel electrophoresis as shown in Table 1. Throughout the purification procedures, the spectrophotometric assay for the enzyme was used. One milliliter of 5% streptomycin sulfate per 5 ml extract was added immediately to remove a major portion of the contaminating nucleic acids. The enzyme precipitated between 60 and 90% with ammonium sulfate yielding a 11.7 fold purification or a 17-fold overall purification. The G-200 Sephadex column was 32 cm in height and 2.5 cm diameter. No loss of activity occurred in this step which yielded a 1.3-fold purification. Tris-HCl buffer, 0.05M, pH 7.5 was used throughout the extraction and purification procedure until

TABLE 1.--Purification of purine nucleoside phosphorylase from vegetative cells of *Bacillus cereus*.

Procedure	ml	u/ml	Total Units	% Recovery	Protein (mg/ml)	Spec. Activ. (Units/mg)	Purif. Ratio (Stepwise)	Tot. Purif. Ratio
Crude extract + Streptomycin Sulfate	1200	44	52,800	100	15	2.9	1	1
16 hrs/4C, followed by centrifugation	1160	41.9	48,640	92.5	9.7	4.2	1.45	1.45
60-90% $(NH_4)_2SO_4$ (ppt. resuspended)	220	214	47,195	89.2	.435	49.2	11.7	17
G-200 Sephadex Chromatography*	522	98.8	51,000	97.5	.15	65	1.3	22.4
Polyacrylamide Gel Electrophoresis*	62.5	121.4	7,600	13.9	.028	6,100	94	2,100

*Corrected for using only 13.6% of the original preparation.

the enzyme was applied to the acrylamide gel column. A 94-fold purification was achieved at approximately 15% recovery on electrophoresis. Note also that the specific activity of the best acrylamide gel fractions are very high, 25% higher than those previously published (19). One protein staining band which corresponded to the enzyme staining band was obtained on analytical gel electrophoresis of the purified vPNPase (Figure 1).

Purine nucleoside phosphorylase from spores of Bacillus cereus was somewhat more difficult to purify than the vegetative cell enzyme. Two series of ammonium sulfate fractionations, followed by treatment with streptomycin sulfate, gel filtration, and two acrylamide gel electrophoresis runs were required as shown in Table 2. Approximately 50% of the enzyme activity was lost on ammonium sulfate fractionation while achieving a purification ratio of 1.5. The object of this step was the concentration of the enzyme.

The Sephadex G-100 column used for gel filtration was 2.5 cm in diameter and 40 cm in height. Up to 60% of the activity of spore PNPase was lost in concentrating the enzyme by ammonium sulfate precipitation after Sephadex filtration. The specific activity of the best acrylamide gel fractions were approximately the same as those which had been reported in the literature. One protein band

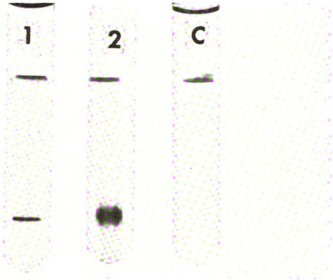


Figure 1.--Analytical acrylamide gel electrophoresis of vegetative cell purine nucleoside phosphorylase of B. cereus obtained by preparative electrophoresis. Approximately 30 units of enzyme were used per tube. The buffer was the standard Tris-glycine, pH 8.3. The upper buffer contained bromphenol blue as an anionic marker. Tube #1 was stained with Amido Schwarz protein stain. Tube #2 was stained for enzyme activity using triphenyl tetrazolium chloride. Tube C was the control containing no enzyme. It was stained for enzyme activity. (The migration markers were decolorized in the staining processes.)

TABLE 2.--Purification of purine nucleoside phosphorylase from spores of *Bacillus cereus*.

Procedure	ml	u/ml	Total Units	% Recovery	Protein (mg/ml)	Spec. Activ. (units/ mg.)	Purif. Ratio (Stepwise)	Total Purif. Ratio
Crude Extract and 45-75% $(NH_4)_2SO_4$	300	209	92,786	100	27	7.75	1	1
Streptomycin Sulfate (1 ml @ 5%/5 ml Sup.)	300	311	99,360	107				
55-90% $(NH_4)_2SO_4$	70	655	45,891	53.8	55	11.9	1.5	1.5
G-100 column, 0-80% $(NH_4)_2SO_4$ dialysis*	42	446	18,700	20.2	12.3	55	4.6	7.1
Polyacrylamide gel electrophoresis #1*	50.8	259.44	13,100	14.1	.88	293	5.3	37.8
Polyacrylamide gel electrophoresis #2**	72	118.68	8,500	9.2	.03	3940	13.4	510

*Corrected for using 50% of preparation.

**Corrected for using 22.5% of preparation.

was obtained in analytical gel electrophoresis, corresponding to the one enzyme activity staining band (Figure 2).

Analytical Gel Electrophoresis

Marked differences were noted in the specific activities of the spore and vegetative cell enzymes. Because this could be a reflection of major structural differences, the enzymes were tested for their relative mobilities in analytical gel electrophoresis. The Rm or relative mobility was defined as the distance traveled by the enzyme sample relative to the distance traveled by the bromphenol blue (anionic) marker. Homogenous enzyme samples prepared by preparative gel were run in duplicate followed by staining for both protein and active enzyme. The enzymes were run in 0.05M Tris-glycine pH 8.3 and 0.002M potassium phosphate pH 8.3. The enzymes had the same Rm value in Tris, but the vegetative cell enzyme was more anionic in the presence of phosphate ions.

In order to determine if the mobility of the enzymes was a function of the phosphate concentration during acrylamide electrophoresis the following buffers were employed: potassium phosphate pH 8.3 at 0.0005M, 0.001M, 0.002M, and 0.01M and the standard Tris-glycine buffer pH 8.3 (Figure 3). As shown in Table 3 and Figure 4, the cell and spore phosphorylases exhibited changes in their electrophoretic mobilities which were dependent on the phosphate concentration, differences in relative mobility

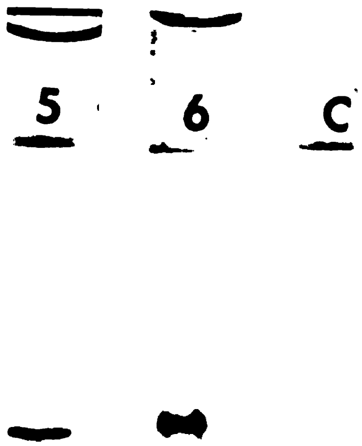


Figure 2.--Analytical acrylamide gel electrophoresis of spore purine nucleoside phosphorylase of *B. cereus* obtained by preparative electrophoresis. Approximately 30 μg of enzyme were used per tube. The buffer was standard Tris-glycine, pH 8.3. The upper buffer contained bromphenol blue as a marker to show migration in the gels. Tube #5 was stained with Amido Schwarz protein stain. Tube #6 was stained for enzyme activity using triphenyl tetrazolium chloride. Tube C was the control containing no enzyme. (The migration markers were decolorized in the staining process.)

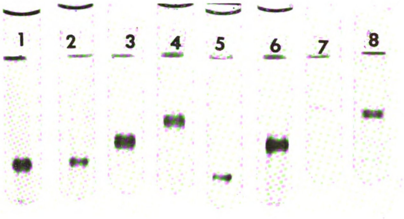


Figure 3.--Comparison of mobility in analytical gel electrophoresis of spore (sPNPase) and vegetative cell (vPNPase) purine nucleoside phosphorylases in various buffer systems. The proteins were stained for enzyme activity. The gel column and bromphenol blue markers were measured prior to development for enzyme activity and the column was remeasured after staining. #1 and 2 vPNPase and sPNPase in 0.05M Tris-glycine, #3 and 4 vPNPase and sPNPase in 0.0005M potassium phosphate, #5 and 6 vPNPase and sPNPase in 0.002M potassium phosphate, and #7 and 8 vPNPase and sPNPase in 0.01M potassium phosphate. All columns were run at pH 8.3, with 2.5 ma current per gel column.

TABLE 3.--Effect of phosphate on spore and vegetative cell purine nucleoside phosphorylases in acrylamide gel disc electrophoresis.*

Phosphate Concentration	Rm (Spore PNPase)	Rm (Veg PNPase)
No phosphate	0.98	0.98
0.0005 M	0.75	0.98
0.001 M	0.69	0.92
0.002 M	0.72	0.86
0.01 M	0.66	0.66

*Approximately 30 µg of protein were placed on each gel column. The upper buffer included bromphenol blue as a marker. The current on each gel was 2.5 milliamperes. The gels were removed from the electrophoresis apparatus and the length of the gel and the distance traveled by the marker band was measured. After staining for enzyme activity, the distance traveled by the enzyme and the length of the gel were measured. From this information, the relative mobility of the enzymes was calculated.

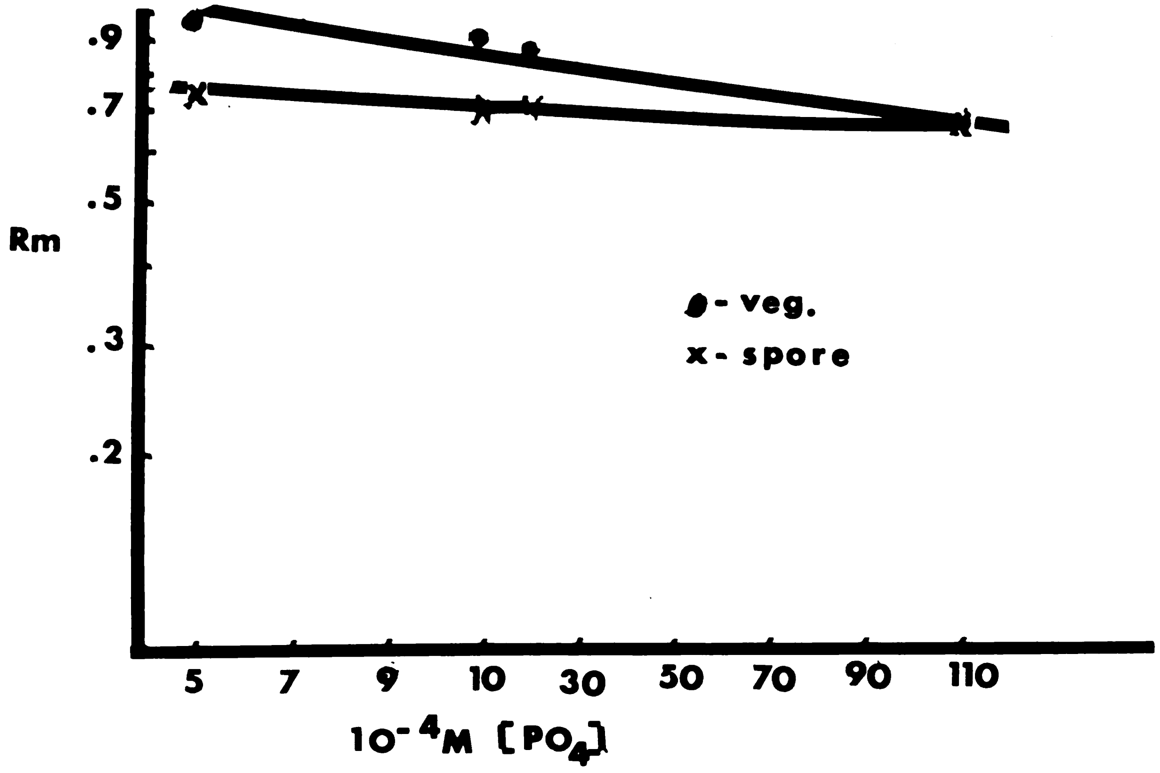


Figure 4.--Log-log plot of the effect of phosphate on the relative mobility of the purine nucleoside phosphorylases in disc gel electrophoresis.

were observed. The spore enzyme migrated at approximately the same rate in all concentrations of phosphate. However, in the absence of phosphate, the migration of the spore enzyme increased to a value equal to that of the vegetative enzyme. It appeared that the mobility of the spore enzyme was not affected by the phosphate in the same way as the vegetative cell enzyme.

Functional Properties

pH Response

The effect of the hydrogen ion concentration on enzymes was studied using the colorimetric assay at 25C and the spectrophotometric assay at 37C. The assays were identical to those described in the materials and methods except the buffers described by Good *et al*. (21) were used. 2-(N-Morpholine)Ethanesulfonic acid$\cdot H_2O$ pK 6.15 and N-Tris(hydroxy-methyl)Methyl-2-Amino-Ethane Sulfonic Acid (TES) pK 7.5 were used in combination at 0.1M to obtain buffers over the range of pH 5.0 to 8.6. This system permitted a wide range of pH values while preventing any possible phosphate concentration effect on the assays which would have occurred using Tris-phosphate buffers over this range. Phosphate was used in the spectrophotometric assay in substrate amounts (0.01M).

The assays at 25C showed a pH optimum of 7.7 based on three assays at each pH for each enzyme (Figure 5).

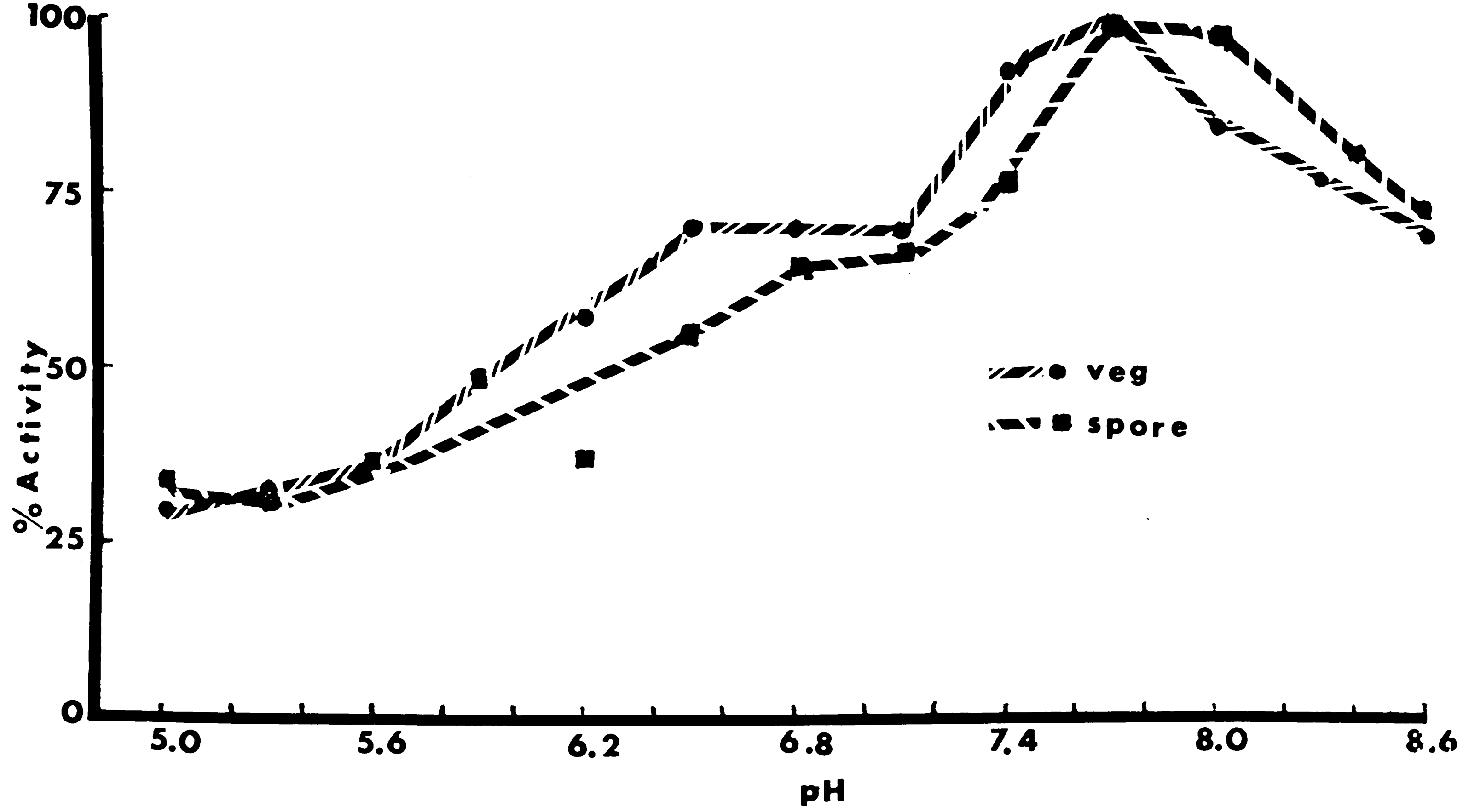


Figure 5.--pH responses of the purine nucleoside phosphorylases using the colorimetric assay at 25C. Enzyme of specific activity over 1,000 was used with an activity of approximately 30 units per milliliter for each assay.

The assays at 37C showed a pH optimum at 8.3 based on two assays at each pH for each enzyme (Figure 6). The optimum pH appears to be a function of the method used to test for the enzyme. In the spectrophotometric system, no activity could be detected at the low pH values. This was due to the pH sensitivity of the xanthine oxidase in the MES buffer system. The two enzymes were similar with respect to their activity and pH response.

Kinetic Parameters

Each of the purified purine nucleoside phosphorylases was assayed with varying concentrations of inosine and constant excess phosphate. They were also assayed with varying concentrations of phosphate and constant excess inosine at pH 7.5.

The spore purine nucleoside phosphorylase had a V_{max} of 182 units at 37C based on Lineweaver-Burke plots (52) for inosine as the substrate and a Km of 7.3×10^{-5}M (Figure 7).

The vegetative cell purine nucleoside phosphorylase at 37C had a V_{max} of 192 units based on the Lineweaver-Burke plots for inosine as substrate. The Km was determined to be 6.7×10^{-5}M for the vegetative cell enzyme (Figure 7).

At 37C, the spore purine nucleoside phosphorylase had a V_{max} of 188 units and Km of 7.2×10^{-3}M with phosphate as limited substrate. The vegetative cell purine

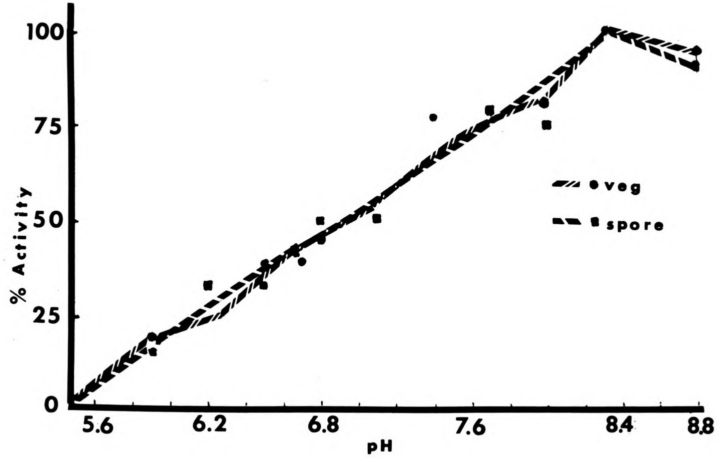


Figure 6.--pH responses of the purine nucleoside phosphorylases using the spectrophotometric assay at 37C. Enzyme samples contained 120 units per millileter with a specific activity over 1,000.

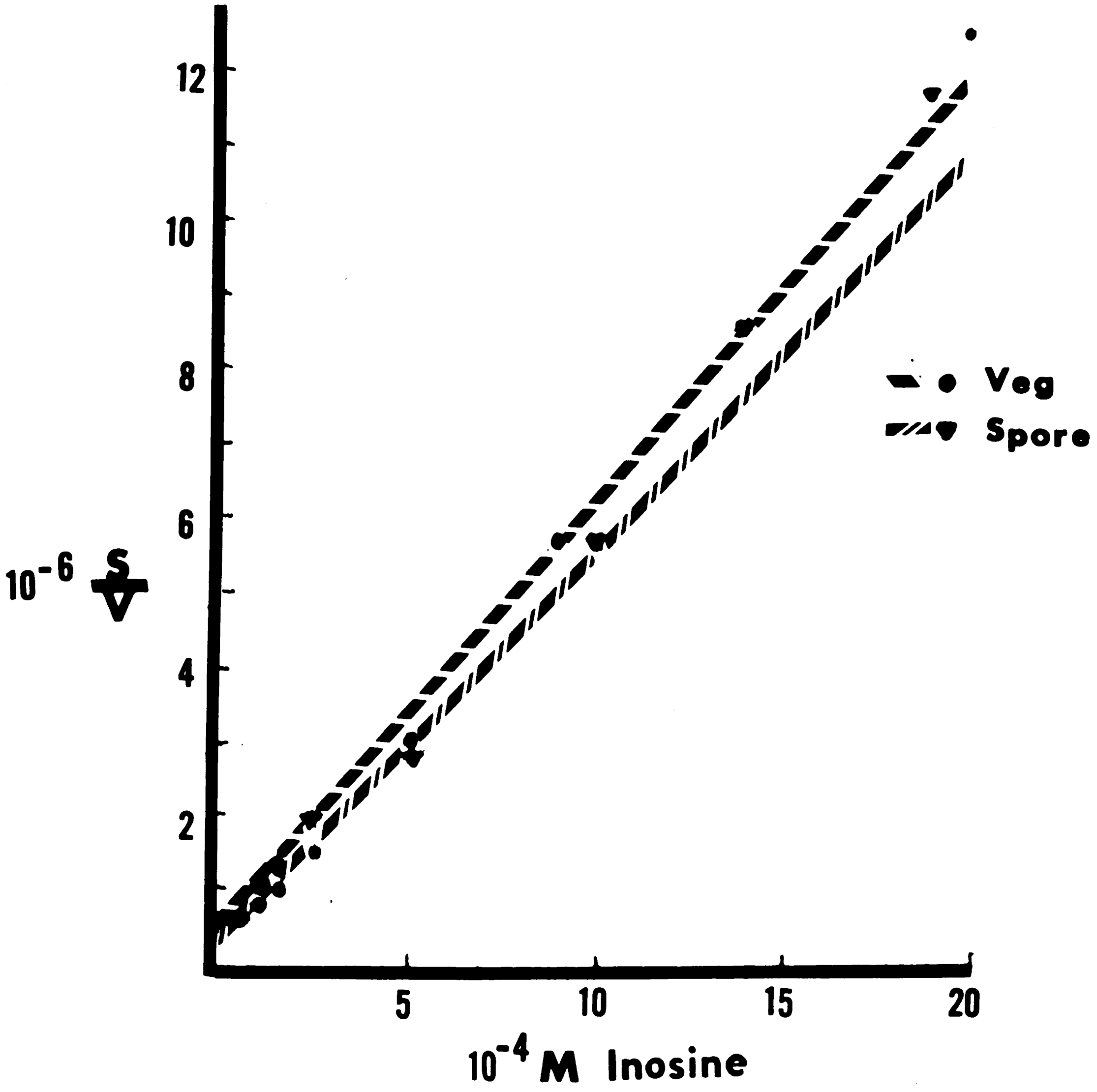


Figure 7.--Lineweaver-Burke plot of the spore and vegetative cell purine nucleoside phosphorylases. The enzymes were measured with the spectrophotometric assay at various concentrations of inosine and excess phosphate. vPNPase: V_{max} = 192 units, Km = 6.7×10^{-5}, sPNPase: V_{max} = 182 units, Km = 7.3×10^{-5}.

nucleoside phosphorylase had a V_{max} of 180 units with a Km of 5.1×10^{-3}M with respect to phosphate (Figure 8). Because of the pronounced effect of phosphate in the electrophoretic mobilities of the enzymes in the electrophoretic mobilities of the enzymes, Hill plots were constructed to determine if more than one substrate molecule bound per molecule of enzyme. The slopes of the Hill plots are shown in Figure 9 to be 1. Therefore, the enzymes do not appear to be allosteric with respect to phosphate. This does not, however, eliminate the possibility of the one phosphate molecule binding at more than one place on the enzyme or in certain cases, more than one phosphate binding to the enzyme.

PHMB Inhibition and Reactivation

Sulfhydryl group involvement at or near the active site of the enzyme, may be demonstrated by blocking them with mercury causing a loss of enzyme activity. Upon the addition of thiols such as mercaptoethanol or dithiothreitol (Cleland's reagent) the thiol groups of the enzyme are regenerated and the activity is recovered (10).

Vegetative cell and spore purine nucleoside phosphorylases were treated with various concentrations of p-hydroxymercuribenzoate (PHMB) to determine an inhibitory concentration of the reagent. The enzymes were subsequently treated with an equal volume of 10^{-2}M PHMB and

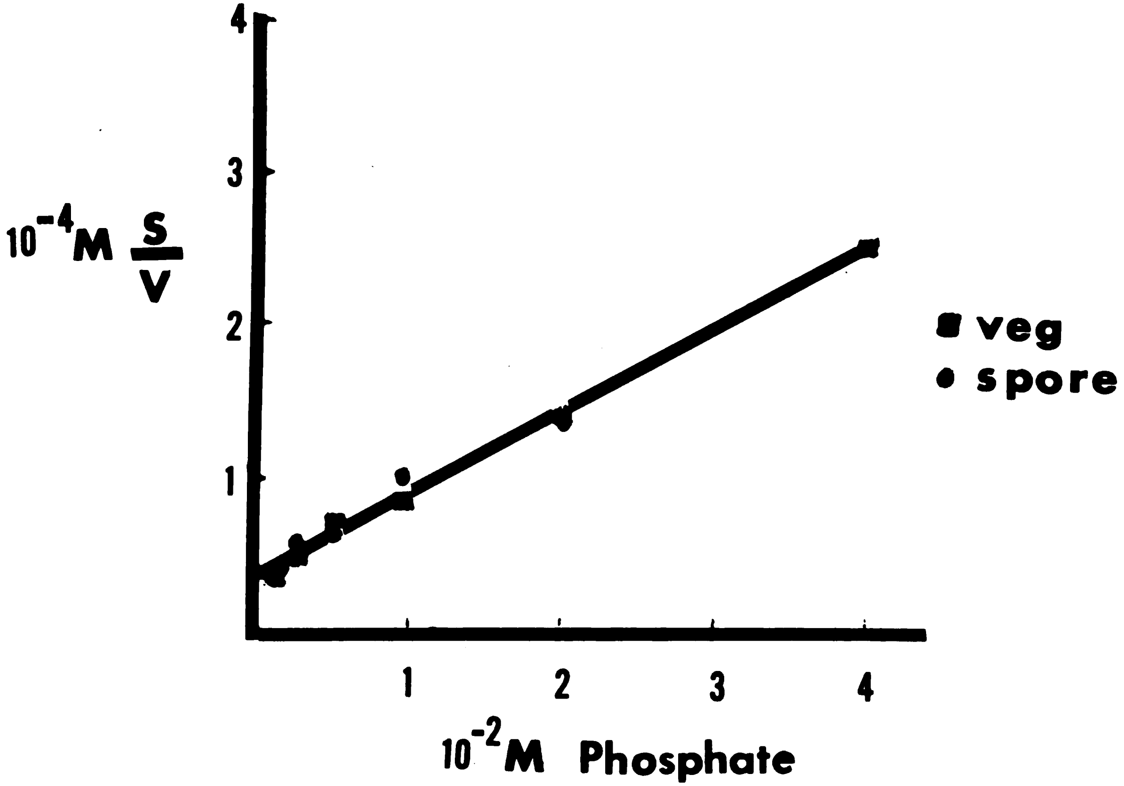


Figure 8.--Lineweaver-Burke plot of spore and vegetative cell purine nucleoside phosphorylases. The enzymes were measured with the spectrophotometric assay at various concentrations of phosphate and excess inosine. vPNPase: V_{max} = 180, Km = 5.1 x 10^{-3}M, sPNPase: V_{max} = 188, Km = 7.15 x 10^{-3}M.

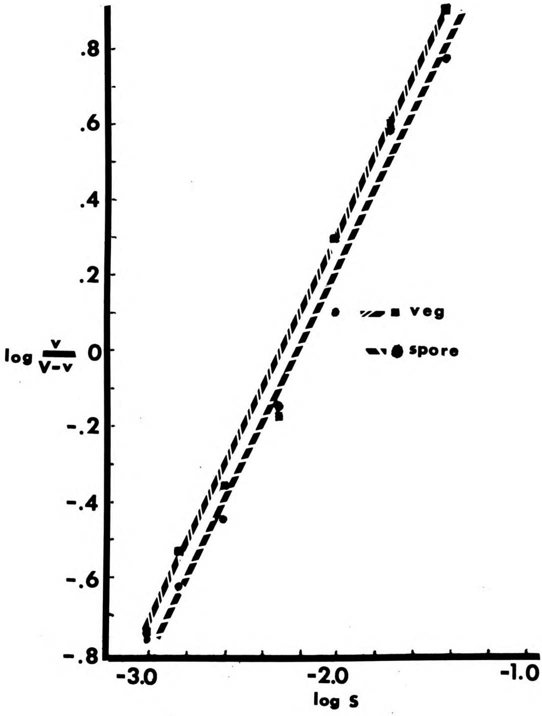


Figure 9.--Hill plots of the purine nucleoside phosphorylases showing a slope of 1.0 for both enzymes with respect to phosphate concentrations.

assayed after five minutes. No activity remained in either crude enzyme preparations or in highly purified fractions from the preparative acrylamide gel column. The enzyme activity was recovered by treatment with Cleland's reagent and mercaptoethanol as recorded in Table 4. From these observations, it can be concluded that there are sulfhydryl groups present in the active site of the enzyme.

Ion Effects

A number of metal ions were tested for their effect on the activities of highly purified vegetative cell and spore PNPases. No effects were observed in the cases of sodium (10^{-1}M), magnesium (10^{-2}M), zinc (10^{-3}M), cobalt (10^{-3}M), ferrous iron (10^{-4}M) or potassium (10^{-1}M). A 1.8 fold increase in activity was observed in the spore enzyme in the presence of 10^{-3}M manganese. A 1.1 fold increase was observed in the case of the vegetative cell enzyme treated with 10^{-3}M manganese.

Calcium ions have an inhibitory effect on the activity of the enzymes. The averages of five sets of assays are presented in Table 5.

Physical Properties

Sedimentation

The sedimentation behavior of the vegetative cell and spore purine nucleoside phosphorylases in the presence

TABLE 4.--Effect of sulfhydryl reagents on spore and vegetative cel purine nucleoside phorphorylases.*

	Spore Enzyme		Vegetative Cell Enzyme	
	Units/ml	% Recovery	Units/ml	% Recovery
Original activity	3	100	8	100
PHMB Treatment	0	0	0	0
Cleland's reagent	2.76	92	4.96	62
Mercaptoethanol	5	100	2.48	31

*Spore and vegetative cell PNPases of specific activity greater than 3,000 units/mg were treated with an equal volume of 10^{-2}M parahydroxymercuribenzoabe and assayed after five minutes at room temperature. The treated enzymes were divided into two 0.1 ml samples. One was treated with 1M Cleland's reagent. The other was treated with 1.28 moles of mercaptoethanol. The preparations were allowed to incubate five minutes at room temperature then assayed.

TABLE 5.--Effect of calcium on spore and vegetative cell purine nucleoside phosphorylase.*

Molar Calcium	Spore PNPase		Vegetative Cell PNPase	
	Units/ml	% Activity Remaining	Units/ml	% Activity Remaining
10^{-6}	119	100	127	100
10^{-5}	119	100	127	100
10^{-4}	119	100	127	100
10^{-3}	103	86	100	82
5×10^{-3}	44	37	32	25
10^{-2}	5.5	13	2.5	2

*Spectrophotometric assays as reported in Materials and Methods were run with 0.5 ml instead of 0.6 ml 0.1M phosphate and the amount of calcium in 0.1 ml necessary to give the proper molarity was added to the cuvette. The reactions were started by addition of the enzyme. The specific activities were 600 units per mg and 2,162 units per mg for the spore and vegetative cell enzymes respectively.

and absence of phosphate was determined by sucrose density gradient centrifugation (57). These experiments were undertaken because of the effects of the phosphate ion on the mobilities of the phosphorylases during electrophoresis.

Sucrose gradients were prepared for each enzyme in

0.05M Tris-HCl
0.05M Tris-HCl and 0.001M potassium phosphate
0.05M Tris-HCl and 0.01M potassium phosphate
0.05M Tris-HCl and 0.05M potassium phosphate

buffers, all at pH 7.5. Two to five sedimentation runs per phosphate concentration were run for each of the two enzymes. On the basis of fourteen determinations, it was found that the sedimentation of the vegetative cell purine nucleoside phosphorylase did not change as a function of phosphate concentration. The data were analyzed and shown to be consistent at the 95% confidence interval. On the basis of five determinations, it was also shown that the spore enzyme has the same sedimentation rate as the vegetative cell enzyme in 0.05M Tris-HCl and 0.05M potassium phosphate pH 7.5. However the spore enzyme and vegetative cell enzyme differ in their sedimentation properties in 0.001M and 0.01M phosphate buffer (Table 6 and Appendix I).

Stokes' Radius

In order to further analyze the effects of phosphate ions on the spore and vegetative cell enzymes, gel

TABLE 6.--Sedimentation data for vegetative cell and spore purine nucleoside phosphorylase based on human hemoglobin. $S_{20,w} = 4.2$

Buffer	Spore PNPase		Vegetative PNPase	
	$S_{20,w}$	Average	Average	$S_{20,w}$
0.05M Tris-Hcl pH 7.5	5.4, 5.5	5.45	5.7	5.6, 5.8
0.001M Potassium Phosphate pH 7.5	6.0, 6.0 6.5	6.17	4.9	4.7, 4.8, 4.9, 5.1, 5.2
0.01M Potassium Phosphate pH 7.5	5.7, 5.8, 6.0, 6.1	5.9	5.1	5.0, 5.0 5.1, 5.2
0.05M Potassium Phosphate pH 7.5	4.9, 5.0 5.1	5.1	4.8	4.5, 4.8, 5.1

filtration experiments were performed (1). The Stokes' radii for the enzymes were calculated from these experiments in

0.05M Tris-HCl
0.001M potassium phosphate and 0.05M Tris-HCl
0.01M potassium phosphate and 0.05M Tris-HCl
and 0.05M potassium phosphate, at pH 7.5

using human hemoglobin (a = 3.08) and cytochrome c (a = 1.42) for markers. The results are given in Table 7 and Appendix II.

The data show that the Stokes' radii of the vegetative cell and spore purine nucleoside phosphorylases are significantly different in 0.05M Tris-HCl pH 7.5 and in 0.05M Tris-HCl and 0.001M phosphate, pH 7.5. The Stokes' radius of the spore enzyme remains constant in these two buffers with an a value of 3.9 nm. The vegetative cell enzyme is the same under all conditions tested at the 99% and 90% confidence levels. The spore enzyme in 0.05M potassium phosphate is the same as the vegetative cell enzyme at the 99% and 95% levels. The critical phosphate level for the configurational change in the spore enzyme is between 0.001M and 0.01M phosphate.

Calculated Molecular Weights

The molecular weights of the vegetative cell and spore PNPases at the various phosphate concentrations were calculated from their respective sedimentation constants

TABLE 7.--Stokes' radius and statistical analysis of data for spore and vegetative cell purine nucleoside phosphorylases in various buffer systems.

Buffer	sPNPase		vPNPase	
	a Values	av	av	a values
0.05M Tris-HCl pH 7.5	3.6, 3.7, 3.9, 4.0, 4.1	3.86	5.2	5.1, 5.1, 5.2, 5.2, 5.4
0.001M Potassium Phosphate pH 7.5	3.8, 3.8, 4.0	3.87	5.0	5.0
0.01M Potassium Phosphate pH 7.5	4.7, 4.9	4.8	5.0	5.0
0.05M Potassium Phosphate pH 7.5	5.4, 5.6, 6.0	5.68	5.1	4.9, 5.1, 5.2

and Stokes' radii assuming a partial specific value of 0.725 cm^3/g. These values were as follows: The molecular weight of the vegetative cell enzyme is 110,000. The molecular weight of the spore enzyme in 0.05M Tris-HCl is 87,000, in 0.001M phosphate is 99,000, and in 0.01 and 0.05M phosphate is 117,000 (Table 8).

Thermal Inactivation

Another possible difference in the vegetative cell and spore purine nucleoside phosphorylases is their relative ability to withstand elevated temperatures over various time periods. Samples of the enzymes were assayed, placed in a regulated water bath, and sampled at designated time intervals. The enzyme remaining at various times was noted as a function of temperature and the composition of the suspending medium.

At 50C in Tris buffer, the purified vegetative cell enzyme had a half life of 10 minutes while the enzyme from spores had a half life of approximately 75 minutes (Figure 10). In the presence of 0.05M phosphate, the heat stability of the spore enzyme was reduced to that of the vegetative cell PNPase.

The thermal stability of the enzyme was also studied at 60C with respect to the calcium ion content in 0.05M Tris-HCl pH 7.5 (Figure 11). At this temperature, the half-life of the vegetative cell enzyme in Tris buffer was 4 minutes while that of the spore enzyme was

TABLE 8.--Calculated molecular weights of purine nucleoside phosphorylases.

Buffer	sPNPase	vPNPase
0.05M Tris-HCl	87,000	110,000
0.001M $PO_4^=$	99,000	110,000
0.01M $PO_4^=$	117,000	110,000
0.05M $PO_4^=$	117,000	110,000

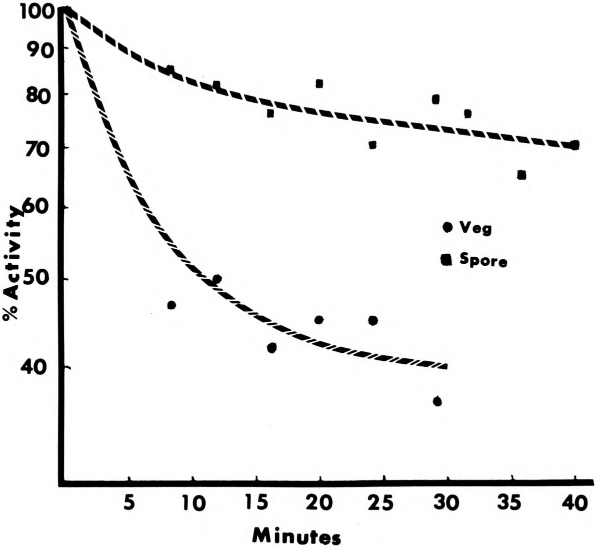


Figure 10.--Thermal inactivation of the purified purine nucleoside phosphorylases at 50C. The enzyme was tested in the absence of phosphate and calcium in 0.05M Tris-HCl buffer pH 7.5. The specific activities of the two enzyme samples was greater than 3,000.

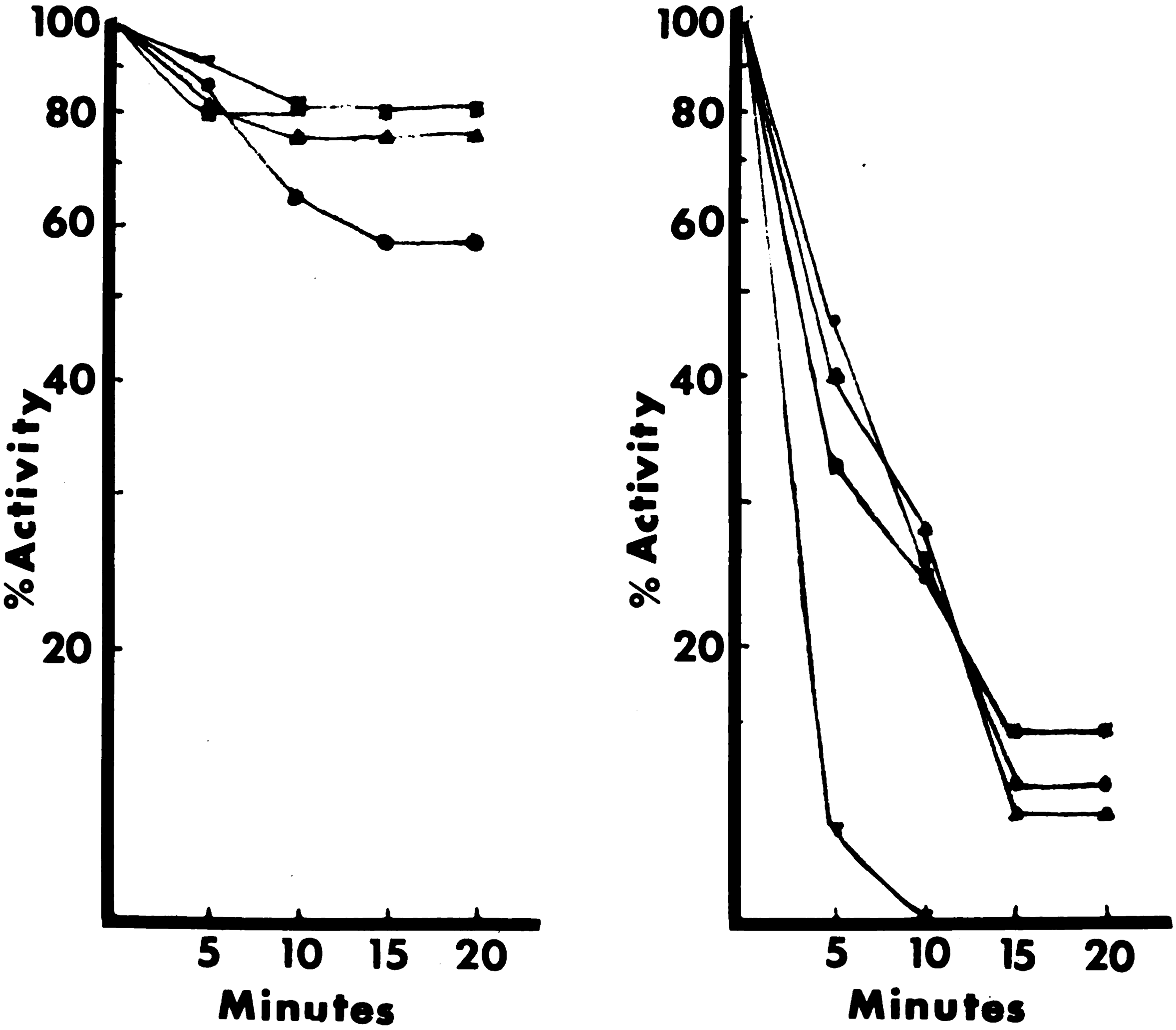


Figure 11.--Thermal inactivation of (a) spore and (b) vegetative cell purine nucleoside phosphorylases at 60C. The effect of calcium on the vegetative cell enzyme is shown. ▲ = Control. No calcium ■ = 5 x 10^{-4}M, ● = 10^{-3}M, and ▼ = 5 x 10^{-3}M calcium.

approximately 30 minutes. Calcium ions have relatively little effect on the heat resistance of the spore enzyme. However, a 50% loss occurred in the stability of the vegetative cell enzyme at 5×10^{-3}M calcium.

Proteolysis of the Vegetative Cell Enzyme

In order to determine if the vegetative cell purine nucleoside phosphorylase could be converted to the spore form by limited proteolysis the vegetative enzyme was treated with purified protease of *Bacillus cereus*. Protease was prepared as reported in Materials and Methods. As shown in a previous section, the Stokes' radius of the spore enzyme 3.9 nm while the vegetative cell enzyme had a radius of 5.1 nm in 0.05M Tris-HCl, pH 7.5. On this basis, if the vegetative cell enzyme was converted to the spore form, a change in Stokes' radius would be observed after limited proteolysis.

Three samples of vegetative cell enzyme were treated with various concentrations of protease then placed on a calibrated G-200 Sephadex column. The a values were 5.0, 5.04, and 4.9. These values were the same or similar to the vegetative cell value of 5.1. Therefore, in spite of the drop in activity as shown by all three samples (Figure 12), the vegetative cell form of the purine nucleoside phosphorylase does not appear to be converted to the spore form by treatment with sporulation-specific protease.

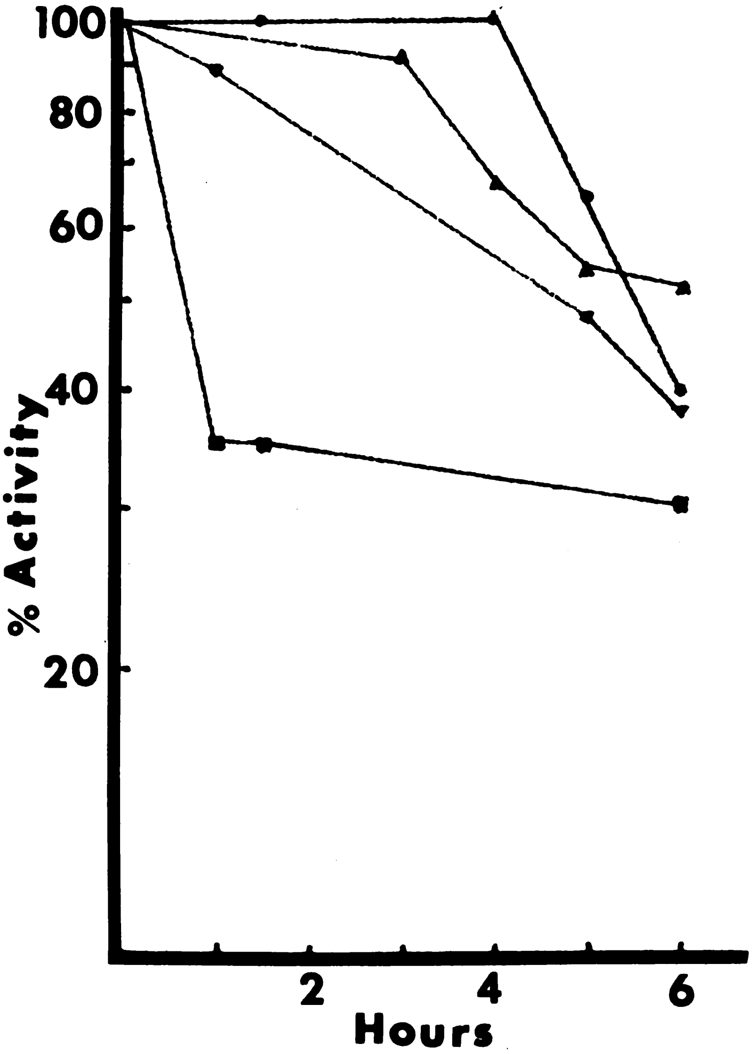


Figure 12.--The effect of protease on vegetative cell purine nucleoside phosphorylase. ▲ = no protease, ▼ = .02 units protease, ● = 0.03 units protease, ■ = 0.04 units protease. Purified enzyme samples, 0.3 ml (SA 5,000) containing approximately 100 u/ml were incubated at 4C for six hours. Samples were assayed at 30 minute intervals for two hours then every hour.

DISCUSSION

The purpose of the research described in this thesis was to further characterize the purine nucleoside phosphorylases (PNPase) from vegetative cells and spores of *Bacillus cereus*. From their specific activities and molecular weights, the turnover numbers of the spore and vegetative cell enzyme were calculated to be 128 and 186 moles of inosine per mole of enzyme per second respectively. The presence of manganese resulted in a pronounced stimulatory effect on the spore PNPase, but very little effect on the vegetative cell enzyme. Taking into account the respective 1.8 and 1.2 fold stimulation by manganese, the turnover number of the two phosphorylases become identical. Although Gardner and Kornberg (19) showed that the synthesis of the enzymes were directed by one genomic unit, the results presented in this thesis show that the enzymes are different in some properties.

A major difference appeared in the effect of phosphate ions on the structure of the two enzymes. This was first observed in disc gel electrophoresis where the mobilities of the two enzymes in the absence of phosphate or in excess phosphate were the same. The movement of an v

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enzyme in acrylamide disc gel electrophoresis is known to be controlled by the Stokes' radius of the molecule and the molecular net charge. In the absence of phosphate, the Stokes' radius of the spore PNPase was smaller than that of the vegetative cell enzyme. Therefore, the vegetative cell enzyme must be more negatively charged. At low phosphate concentrations (below 10^{-3}M), the Stokes' radius of the spore enzyme was only 80% of that of the vegetative cell enzyme. Since no change of size was observed in the vegetative cell enzyme, the changes in its relative mobility over the concentration range of phosphate studied must have been due to a decreasing negative charge on the molecule. The vegetative cell enzyme probably became less anionic in high phosphate concentrations by exposure of positive groups. In high concentrations of phosphate, the size of the spore enzyme increased. There was an unknown charge effect on the spore enzyme, but it was equal to the charge effect on the vegetative cell enzyme in 0.01M phosphate.

The Stokes' radius of an enzyme is that value for a hypothetical spherical molecule displaying similar hydrodynamic properties determined by the ability of the molecule to diffuse a certain distance at an average velocity under the same experimental conditions. The Stokes' radius of the spore enzyme was shown to be significantly different from that of the vegetative cell enzyme

in low concentrations (10^{-3}M) or in the absence of phosphate. At higher concentrations of phosphate (greater than 10^{-2}M), the spore and vegetative cell enzymes appeared to have the same Stokes' radius. Thus, there appeared to be a critical level of phosphate required for the conversion of the spore enzyme to a form similar to the vegetative cell enzyme. This level corresponded well to the Km for phosphate as substrate (7.15×10^{-3}M). The significance of the changes which occur near the Km for phosphate may be due to substrate binding to the enzyme. However, on the basis of the Hill plot, there was nothing unique with respect to the phosphate binding at the active site.

A further effect of phosphate was shown by the changes in heat resistance of the spore enzyme. The spore enzyme was shown to be more heat stable in the absence of phosphate. In the presence of phosphate, the heat resistance was equal to that of the vegetative cell enzyme. The same effect was shown using the analog arsenate instead of phosphate (Sadoff, unpublished results).

The information obtained from the sedimentation constants and the Stokes' radius determinations permitted an approximation of the molecular weights. The vegetative cell enzyme had the same molecular weight regardless of its environment with respect to phosphate ions (110,000). The spore enzyme (88,000) appeared to increase its

molecular weight by 30,000 in the presence of phosphate concentrations higher than the Michaelis constant. The molecular weight of the spore enzyme (117,000) was similar to that of the vegetative enzyme (110,000). The difference is within the error of the estimate. The intermediate value (99,000) represented a weight average molecular weight between the two forms.

An explanation for the apparent changes in molecular weight of the spore enzyme in the presence of phosphate was developed and is based on the ability of the molecule to disaggregate. It is proposed that the vegetative cell and spore enzymes are each composed of four subunits of approximately 30,000 molecular weight. The PNPases as initially synthesized in cells or spores are identical. This is consistent with their control of synthesis by one cistron. However, during the development of the spore, some structural modification (viz. proteolysis, hydrolysis, or removal of a functional group) occurs which permits the molecule to exist in a lower state of aggregation. In the absence of phosphate and arsenate, the predominate form consists of three subunits and has a higher intrinsic heat resistance than the aggregate containing four subunits. In this respect the spore enzyme resembles the glucose dehydrogenase of *Bacillus cereus* which is more heat resistant when in a disaggregated state (78). In higher phosphate concentration, a

redistribution of subunits occurs which yields the four subunit spore PNPase. It is identical to the vegetative enzyme except for its lower turnover number. The addition of manganese to the four subunit form of the spore enzyme results in a molecule which has identical activity to the vegetative cell PNPase. Although this hypothesis is dependent upon unusual stoichiometry which has never been previously reported for any other enzyme, it is consistent with the data.

A 50% loss of heat stability occurred when the vegetative cell enzyme was in 5 x 10^{-3}M calcium chloride. Relatively little effect was observed on the heat resistance of the spore enzyme at any calcium concentration. Both enzymes were inhibited by 10^{-2}M calcium chloride. Thus the dormancy of the spore PNPase may be the result of the high calcium concentration in spores.

The two PNPases were expected to have similar physical and chemical properties because they were products of the same cistron. Both enzymes were inhibited by p-hydroxymercuribenzoate and reactivated by sulfhydral reagents showing the presence of -SH groups at the active sites of the enzymes. The effect of the change in pH on the two enzymes was also shown to be very similar. There appeared to be a particularly negative effect of the morpholine in the Good's buffers at low pH as shown by low activities in the spectrophotometric assays. This

could be an effect on the xanthine oxidase rather than on the PNPases. The Km of the two enzymes with respect to both phosphate and inosine were shown to be very similar.

The experiments involving limited proteolysis of vegetative PNPase were an attempt to convert that enzyme into the spore form. The differences between the vegetative cell and spore PNPases were not due to proteolytic cleavage of the vegetative enzyme.

It may be concluded that despite all similarities observed in these enzymes, including their genetic control by a single cistron, the enzymes appear to be different aggregations of the same kinds of material. The effect of the phosphate may be a result of the structural modification of the active center, revealing E- amino groups from lysine or histidyl groups thus giving the molecule a net positive charge.

SUMMARY

The purine nucleoside phosphorylases of Bacillus cereus spores and vegetative cells were each purified to a state of electrophoretic homogeniety by ammonium sulfate fractionation, gel filtration, and preparative gel electrophoresis. The enzymes had previously been shown to originate from one cistron and were similar in many properties. They were identical in their pH-activity spectra with optima at 8.3 and 7.7 depending on the method of assay. The molecular weights of the enzymes in the presence of excess phosphate were approximately 110,000. The Michaelis constants for phosphate were 7.3×10^{-3}M and 5.1×10^{-3}M for the spore and vegetative cell PNPases. The Michaelis constants for inosine were 7.3×10^{-5}M and 6.7×10^{-5}M for the spore and vegetative cell PNPases. Both PNPases were inactivated by mercuration and reactivated by Cleland's reagent or mercaptoethanol (Sulfhydryl reagents). The activities of the enzymes were enhanced in the presence of manganese, though to different extents. The turnover numbers for the spore and vegetative cell enzymes were calculated to be 128 and 186 moles of inosine per mole of enzyme per second,

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respectively. In low concentrations of phosphate, the vegetative cell enzyme was more anionic than the spore enzyme during gel electrophoresis. The Stokes' radii of the enzymes were determined by the method of Ackers (1) using calibrated Sephadex G-200 columns. The sedimentation constants were obtained from the mobilities of the enzymes by centrifugation in sucrose density gradients. The Stokes' radii and sedimentation constants of the vegetative cell enzyme preparations were constant over a wide range of phosphate concentrations. These parameters of the spore enzyme were concentration-dependent with respect to phosphate. The molecular weight of the spore enzyme increased from approximately 90,000 to 120,000 while that of the vegetative cell enzyme remained at 110,000 as the phosphate ion concentration was increased from zero to 0.05M. The spore and vegetative cell enzymes were identical at phosphate concentrations above the Michaelis constants for phosphate. The half-life of the spore PNPase at 50C was approximately 75 minutes in the absence of phosphate, but decreased to 10 minutes in 0.05M phosphate. This was equal to the stability of the vegetative cell PNPase in the presence or absence of phosphate. The vegetative cell enzyme is more sensitive to the inhibitory effects of calcium. The process which converts the vegetative PNPase to the spore form does not appear to be simple proteolysis.

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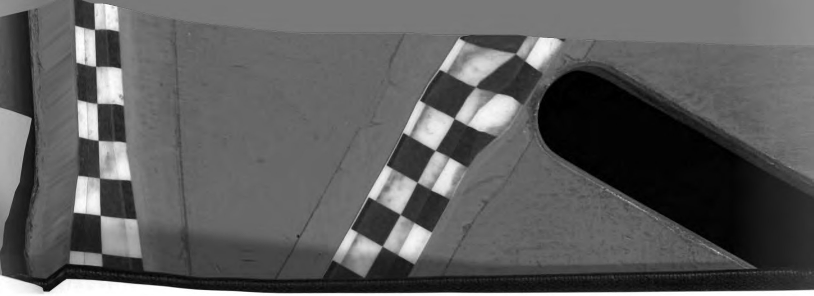

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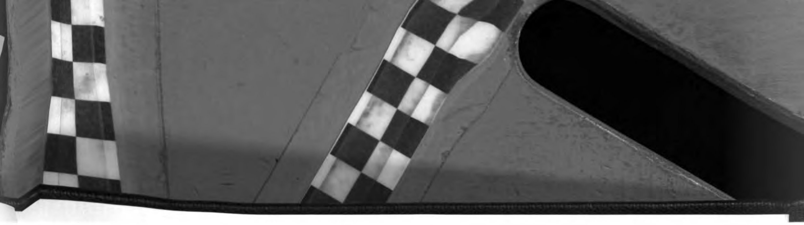
APPENDICES



APPENDIX I

Statistical Analysis of $S_{20,w}$ Results

	Confidence Levels		
	99%	95%	90%
All vPNPase	5.1 ± .9	5.1 ± .7	5.1 ± .5
All vPNPase+ 0.05M Tris-HCl and 0.05M $PO_4^=$	5.1 ± .9	5.1 ± .5	5.1 ± .4
0.05M Tris and 0.05M PO_4 sPNPase	5.16 ± .59	5.16 ± .36	5.16 ± .27
0.001M PO_4 sPNPase	6.2 ± 1.6	6.2 ± .7	6.2 ± .48
0.001M PO_4 vPNPase	5.0 ± .46	5.0 ± .28	5.0 ± .21
0.01M PO_4 sPNPase	5.9 ± .49	5.9 ± .26	5.9 ± .2
0.01M PO_4 vPNPase	5.1 ± .29	5.1 ± .16	5.1 ± .1

APPENDIX II

Statistical Analysis of Stokes' Radius Data

Buffer	sPNPase			vPNPase		
	Confidence Levels					
	99%	95%	90%	99%	95%	90%
0.05M Tris-HCl	3.86 ± 1.45	3.86 ± 0.88	3.86 ±0.66	5.2 ±0.25	5.2 ±0.15	5.2 ±0.11
0.001M $PO_4^{=}$	3.87 ± 1.7	3.87 ± 0.76	3.87 ±0.51		5.0	
0.01M $PO_4^{=}$	4.8 ± 6.3	4.8 ± 1.27	4.8 ±0.63		5.0	
0.05M $PO_4^{=}$	5.7 ± 1.7	5.7 ± 0.76	5.7 ±0.51	5.07 ± 2.2	5.07 ±0.9	5.07±0.6