PHYSICAL AND CATALYTIC PROPERTIES OF THE PURINE NUCLEOSIDE PHOSPHORYLASES ISOLATED FROM VEGETATIVE CELLS AND SPORES OF BACILLUS CEREUS

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY
RICHARD WILLIAM GILPIN

1970

This is to certify that the

thesis entitled

Physical and Catalytic Properties of the Purine Nucleoside Phosphorylases Isolated from Vegetative Cells and Spores of *Bacillus Cereus*

presented by

Richard William Gilpin

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Microbiology

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Date May 14, 1970

O-169

ABSTRACT

PHYSICAL AND CATALYTIC PROPERTIES OF THE PURINE NUCLEOSIDE PHOSPHORYLASES ISOLATED FROM VEGETATIVE CELLS AND SPORES OF *BACILLUS CEREUS*

By

Richard William Gilpin

The purine nucleoside phosphorylase from vegetative cells and spores of *Bacillus cereus* T was purified to electrophoretic homogeneity by ion exchange chromatography and polyacrylamide gel electrophoresis. The specific activity of these preparations was higher than those previously reported. Phosphate ion caused an increase in the thermal stability of both purified enzymes whereas inosine had no effect. The catalytic properties of the two enzymes were similar and indicated an ordered, sequential reaction mechanism where inosine was bound before phosphate. The Michaelis constants (inosine as substrate) for the vegetative cell and spore enzymes were 4.6×10^{-4} M and 7.0×10^{-4} M respectively. The Michaelis constant for phosphate was 1.5×10^{-3} M for the vegetative cell enzyme and 1.3×10^{-3} M for the spore enzyme. Both enzymes had similar turnover numbers and the specific

activities of the enzymes appeared to be constant at varying protein concentrations. Negative homotropic effects were proposed since the Lineweaver-Burk plots with inosine as the changing fixed substrate and phosphate as the variable substrate showed downward curvature at high phosphate concentrations.

Sucrose density gradient studies indicated that spore purine nucleoside phosphorylase (PNPase) increased from a sedimentation velocity of 5.3 S in the absence of phosphate to a value of 5.7 S in the presence of 10 mM potassium phosphate. The increase in sedimentation velocity may have been irreversible. The vegetative cell PNPase had a sedimentation velocity of 5.5 S in the presence or absence of phosphate ion. The calculated minimal molecular weight of spore PNPase was 46,300 in the absence of phosphate, and 92,600 in the presence of 10 mM phosphate. The vegetative cell enzyme had a molecular weight of 88,300 and 91,600 in the presence and absence of phosphate respectively. The subunit size for both enzymes was 24,000 $\pm$10%. The conformational structure of the spore enzyme was more compact in the absence of phosphate ion. Dimerization of the spore enzyme to a molecular weight approximating the size of the vegetative cell enzyme was accompanied by a change in conformation.

Although the synthesis of both the vegetative cell and spore enzymes was directed by the same genomic unit, the enzymes differed in their size and shape when isolated in the absence of phosphate ion.

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology
and Public Health

1970

ACKNOWLEDGMENTS

I wish to thank my advisor, Dr. Harold L. Sadoff, for his excellent support and guidance during this investigation. I am grateful to George M. Stancel, Department of Biochemistry, for his assistance and counsel in the sedimentation equilibrium studies. I thank Andrew E. Seer, Jr., Department of Chemistry, for construction of the preparative disc-gel electrophoresis apparatus. Appreciation is also extended to James C. Johnson, Department of Biochemistry, for his informative discussions concerning subunit analysis.

I also wish to thank Dr. Ralph N. Costilow, and other staff members of the Departments of Microbiology and Biochemistry for their guidance.

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INTRODUCTION

Members of the Bacillaceae are able to undergo a morphogenesis to form resting spores. Recently, this process has been compared to an abortive cell division (28). It is stringent with respect to both the metabolic state of the cells and their mineral nutrition. That is, certain molecular species of cations either enhance or inhibit sporulation (40, 50, 55, 97). Spores are metabolically dormant refractile bodies which are able to withstand extremes in environmental conditions much better than the vegetative cells from which they are derived. In suitable environments, spores germinate and resume vegetative growth. Thus, spore-forming organisms must be taken into consideration in processes which achieve sterilization by heating, drying, or chemical means.

Scientific interest in spores was initially directed toward finding methods to decrease their survival during the processing of canned foods. More recently, because of the striking morphogenesis which occurs, sporulation has been studied as a model of cellular differentiation.

Intact spores are heat resistant and thus it may be assumed that their most labile constituents, proteins,

must also be heat stable *in vivo*. Spore proteins would appear to be excellent models for study of the mechanism of heat resistance at the molecular level. In such studies, enzymes are usually the proteins of choice because they are easily detected by their catalytic properties and they can be purified to homogeneity. Comparisons between the physical and chemical properties of vegetative cell and spore enzymes have revealed differences which must be related to mechanisms by which spores achieve heat resistance. The significance of these differences in relation to spore properties is just beginning to be understood.

Gardner and Kornberg (20) found that the synthesis of the purine nucleoside phosphorylase (PNPase) enzymes from vegetative cells and spores of *Bacillus cereus* var. *terminalis* was directed by the same genomic unit. These authors concluded that the two enzymes had similar catalytic and physical properties. A careful examination of their data, however, showed that distinct differences existed between the two PNPases. Therefore, further studies were made in this laboratory.

Engelbrecht and Sadoff (16) found that pure spore and vegetative cell PNPases differed in their physical and catalytic properties depending on the phosphate ion concentration of the surrounding environment.

Further characterizations of the physio-chemical differences between the cell and spore enzymes are reported in this dissertation.

LITERATURE REVIEW

Metabolism and Sporulation

In batch cultures, the sporulation of Bacillus species occurs at the end of exponential growth when cells are in a metabolic shift-down condition. Control of sporulation is not well understood, but the availability of glucose and the buildup of metabolic products from the fermentation of glucose, such as acetate and poly-B-hydroxybutyrate, seem to be important. Glucose is a non-specific repressor of sporulation and may cause catabolite repression of the sporulation-specific enzymes (44, 80). Acetate, derived from the metabolism of substrates or when added exogenously, induces the glyoxylate cycle in bacilli in the absence of glucose (25).

The tricarboxylic acid (TCA) cycle enzymes have been found at higher levels in sporulating cells than in log phase cultures (6, 13, 89), which are usually devoid of aconitase activity (89). It would appear that these enzymes function in the sporangium during the course of sporulation and, when coupled to oxidative phosphorylation, supply energy for the sporulation process. Spore extracts have no detectable TCA cycle activity. Thus it would appear that this metabolic

cycle is a well compartmentalized function during sporulation.

Protein Turnover During Sporulation

Protein turnover occurs during sporulation. In Bacillus subtilis sporulating in minimal medium (57) or in supplemented nutrient broth (84), protein turnover during sporulation was 18% per hour. Proteins were conserved during exponential growth (84) and little or no turnover occurred. Genetic (83) and kinetic data (2, 19, 64, 93) have indicated that the protein turnover occurs in the sporangium during sporulation.

Ordered Events During Sporulation

Sporulation occurs in a temporal sequence of events after the derepression of the TCA cycle. The time course for both morphogenesis (66) and characteristic enzyme formation (13, 73) has been noted. Sporulation commences in bacilli at the end of exponential growth and is complete in 6 to 8 hours. Studies of particular interest have been made of the onset of protease activity (5), alkaline phosphatase activity (63), glucose dehydrogenase activity (3), refractility (72), synthesis of dipicolinic acid (69), calcium ion uptake (72), and heat resistance (94). A metabolic or genetic block at any point in the sequence of sporulation blocks all subsequent sporulation events once the commitment to sporulation has been made.

Heat Resistance

Heat resistance is one property of bacterial spores and it is easily measured. Spore enzymes are usually heat stable *in vivo* but become labile when removed from the resting spore. A few exceptions have been found, however. The reduced nicotinamide-adenine-dinucleotide oxidase from spores of *Clostridium botulinum* (22) and the catalase from spores of *B. cereus* (75) appeared to be intrinsically stable. In general, the mechanism of heat resistance of spore proteins is unknown.

Various authors, in attempting to explain heat resistance, have suggested that spore and vegetative cell enzymes of homologous activity are uniquely different proteins (23, 24). If this were the case, the cell genome would have to possess a large sporulation-specific complement. This "spore genome" has not been found. One specific cistron per enzyme directed the synthesis of both vegetative cell and spore PNPase (20), alanine dehydrogenase (56), and DNA polymerase (17). Presumably most spore enzymes are coded by the same segment of the chromosome which directs the synthesis of the corresponding vegetative cell protein and some mechanism exists to render them heat resistant (3, 5, 41).

Spore enzymes may be heat stable due to their attachment to particles in the intact spore. Alanine racemase of *B. cereus* was found to be stable when it

was attached to spore wall material (85, 86). Ribosidase was also thought to be stable for this reason (4, 71). Alanine racemase (85), catalase (58), and adenosine deaminase (71), which are active in the intact spore (48), were found in either the spore coat or the exosporium. If this is the case, the aggregates must decompose on disruption or germination of the spores, rendering most of the proteins labile.

The ionic environment of a protein must play a role in its heat stability. It is known that spores containing high levels of divalent cations tend to be heat stable, whereas those grown in media deficient in these ions tend to be heat labile (94). The effect of the ionic environment on heat resistance can also be shown with purified enzymes. Glucose dehydrogenase from spores of B. cereus was found to be heat stable due to a reversible dimer-monomer interconversion promoted by hydrogen ions, with the monomer showing more thermal stability (3, 76). This enzyme could be further stabilized by increasing concentrations of Group IA cations. The fructose 1,6-diphosphate aldolase from spores of the same organism was activated and stabilized specifically by increasing concentrations of calcium (77).

A close relationship between protease activity and sporulation has been described by several authors (5, 51, 60, 61, 79, 82). The activity of protease may

result in many enzymes being modified or degraded during sporulation. That is, proteolytic activity may not be restricted to protein turnover in the sporangium. The altered enzyme forms resulting from proteolysis of the "vegetative protein" may acquire the attribute of heat resistance in the environment of the spore. The fructose 1,6-diphosphate aldolase from vegetative cells of B. cereus appeared to be cleaved by a limit-protease during sporulation to produce an enzyme which was smaller than its vegetative cell counterpart (77). The modified aldolase in the presence of calcium was heat resistant. Protease-negative mutants of B. subtilis were either asporogenous or oligosporogeneous (57, 79, 82). No studies have been made of the heat resistance of enzymes from these types of mutants. When a protease inhibitor such as L-cysteine was added to the medium of a sporulating culture, the intracellular degradation of protein was delayed for many hours and the final yield of spores was much lower than normally found (57). When chloramphenicol was added to a sporulating culture at levels that inhibited protein synthesis, protein degradation and sporulation were prevented (84). The chloramphenicol may have prevented the synthesis of protease or prevented the synthesis of a protein activator for protease. Kornberg (42) suggested that protein synthesis may be required during sporulation to depress the level of a

protease inhibitor(s) such as L-cysteine. Once protein degradation started, the addition of chloramphenicol did not alter its rate although protein synthesis and sporulation were completely blocked (84). Proteolysis might account for the size differences which have been noted between the vegetative cell and spore purine nucleoside phosphorylases found in B. cereus (16).

Purine Nucleoside Phosphorylase

Purine nucleoside phosphorylase (PNPase, EC 2. 4. 2. 1) occurs in vegetative cells and spores of B. cereus T, from which it can be readily isolated. This enzyme can also be induced to three-fold higher concentrations in vegetative cells by one of its substrates, inosine (20). PNPase catalyzes the reaction; purine nucleoside + orthophosphate ⇌α-D-ribose-1-phosphate + purine. Interest in this enzyme was stimulated by its possible role in germination induced by inosine.

B. cereus T spores can be germinated in sodium phosphate buffer containing inosine or inosine plus L-alanine (74). Krask and Fulk (43) found a nucleoside phosphorylase in extracts of B. cereus that cleaved adenosine or inosine in the presence of inorganic phosphate to produce ribose-1-phosphate and purine base. A phosphoribomutase and an adenosine triphosphate ribokinase were also found. These authors concluded that these enzymes could function during germination by synthesizing phosphate esters which would

ultimately yield ATP. It has been shown that the viability of stored erythrocytes can be restored by the addition of nucleosides which leads to the resynthesis of phosphate esters and ATP (8). Spores of many bacilli can cleave adenosine to free base and ribose (46, 47). Spores of B. cereus T were able to degrade adenosine, guanosine, inosine, xanthosine, adenylic acid, cytidine, and uridine (46). Powell and Hunter (70, 71) found that other strains of B. cereus, which germinated more efficiently with inosine instead of adenosine, could hydrolyze ribosides at variable rates. These data suggested a role for nucleoside phosphorylase in spore germination. However, Gardner and Kornberg (20) using a mutant of B. cereus T containing low levels of PNPase activity found that germination was as rapid and complete in inosine as the wild type. This finding contradicts the hypothesis of Krask and Fulk (43) and reopens the question of the role of inosine and PNPase in riboside-induced germination.

The first nucleoside phosphorylase was discovered in rat lever (32, 33, 34, 35). The enzyme catalyzed the reaction; hypoxanthine + ribose-1-phosphate ⇌ inosine + inorganic phosphate. The reaction equilibrium favored the production of inosine. PNPase which will degrade adenosine (14, 39, 46, 47) or inosine and guanosine (27, 58) has also been isolated. This enzyme has been found in human erythrocytes (37, 92), chicken liver (65),

yeast (27), Escherichia coli (58), and B. cereus (46, 47, 48). The metabolic significance of this enzyme is unknown. The ease with which it can be purified makes it useful as a model enzyme in the study of protein differences between vegetative cells and spores of B. cereus.

Gardner and Kornberg (20) compared the PNPases from vegetative cells and spores of B. cereus and concluded that the two enzymes were under control of the same genomic unit. Both enzymes appeared to have similar catalytic and physical properties, but there were subtle differences which prompted further study.

Engelbrecht and Sadoff (16) found that pure spore and vegetative cell PNPases differed in their physical properties depending on the phosphate ion concentration of the surrounding environment. In the absence of phosphate ion spore PNPase had an estimated molecular weight of 87,000 which increased to 117,000 in the presence of 10 mM phosphate. Vegetative cell PNPase had a molecular weight of 110,000 regardless of the phosphate concentration. Spore PNPase also had a 7-fold higher thermal stability than the vegetative cell enzyme in the absence of phosphate. When the phosphate ion concentration was increased to 10 mM, the thermal stability of the spore PNPase decreased to a value equivalent to that of the vegetative cell PNPase which did not

change. Engelbrecht and Sadoff (16) proposed that spore PNPase underwent aggregation in phosphate ion concentrations greater than the K_m for phosphate as substrate (7.2×10^{-4} M). Vegetative cell PNPase was assumed to be unaffected by phosphate.

METHODS

Growth of Vegetative Cells and Spores of Bacillus cereus

Bacillus cereus T (76) was grown in modified G-medium (26). Three liters of exponentially growing cells were used to inoculate a 100 liter fermenter using the step-culture method of Sadoff, et al. (76). Vegetative cell cultures were grown at 30 C to mid-logarithmic phase (0.7 optical density at 620 nm on a Bausch and Lomb Spectronic 20) in 4 hr. Cultures grown for 21 hr yielded greater than 95% free spores. The cells and spores were harvested from the 100 liter fermenter by cooling the culture to 14 C and collecting the cells with a Sharples continuous flow centrifuge. The cell pastes were stored at -15 C.

Spectrophotometric Assay of Purine Nucleoside Phosphorylase

PNPase cleaves inosine in the presence of inorganic phosphate to yield hypoxanthine and ribose-1-phosphate. A coupled spectrophotometric assay of the enzyme was utilized (35) in which hypoxanthine was oxidized to uric acid by xanthine oxidase. The rate of increase in absorbance due to uric acid formation was followed at

290 nm. The assays were performed at 37 C in a Perkin-Elmer Model 124, double beam diffraction grating spectrophotometer coupled to a Sargent Model SL recorder. Alternatively, a Beckman Model DU spectrophotometer coupled to a Sargent Model SL recorder was used.

The standard assay system contained the following reactants: inosine, 2 mM; potassium phosphate buffer, pH 7.5, 30 mM; xanthine oxidase, 0.01 unit; and 1 to 10 units of PNPase. The final assay volume was 1 ml in 1 cm light path cuvettes. All essay components except PNPase were preincubated for 10 min at 37 C to remove trace amounts of hypoxanthine. For the kinetic studies all enzyme and substrate dilutions were made in 50 mM trishydroxymethylaminomethane (Tris)-HCl, 10 mM 2-mercaptoethanol buffer, pH 7.5. This buffer was used throughout the study and was designated the standard buffer.

One unit of PNPase activity was that amount of enzyme needed to catalyze the hydrolysis of 1 μmole of inosine per hr at 37 C. Specific activity was calculated as units of PNPase activity per mg protein.

Xanthine oxidase was prepared from fresh unpasteurized Jersey cow milk by the procedure of Gilbert and Bergel (21). The purified protein was kept at 2 C as a 65% ammonium sulfate precipitate in 200 mM potassium

phosphate buffer, pH 6.0, and only 35% loss of activity was found after ten month's storage.

One unit of xanthine oxidase was that amount of enzyme needed to catalyze the oxidation of 1 μmole of hypoxanthine per min at 25 C.

Measurement of Protein Concentration

Protein concentrations were estimated routinely by the method of Warburg and Christian (95). The protein contents of highly purified PNPase preparations were determined by the method of Lowry, et al. (54).

Protein Concentration by Ultrafiltration

Dilute solutions of PNPase were concentrated in a Diaflo ultrafiltration cell with type UM-10 ultrafiltration membranes (Amicon Corporation, Lexington, Ma.). A positive pressure of 50 psi was maintained over the membrane with compressed nitrogen.

Preparation of DEAE-cellulose for Column Chromatography

One kilogram of Whatman DE-52 DEAE-cellulose was suspended in 5 liters of distilled water and the fine particles were removed by decantation. The exchanger was filtered over Whatman #1 filter paper in a Buchner funnel and suspended in 4 liters of 0.5 N HCl. The suspension was stirred for 30 min and then filtered

and washed with distilled water until the effluent pH was 4.0. It was then stirred into 4 liters of 0.5 N NaOH for 30 min, filtered, and subjected once more to this treatment. The DEAE-cellulose was washed with distilled water until an effluent pH of 7.0 was obtained. It was then suspended in 4 liters of 240 mM NaCl and the pH of the mixture was slowly adjusted to 7.5 using 0.24 N HCl. The ion exchanger was then filtered and equilibrated in 240 mM Tris-HCl, 10 mM 2-mercaptoethanol buffer, pH 7.5.

Preparative Polyacrylamide Disc-gel Electrophoresis

The apparatus used to purify the PNPase enzymes was constructed according to the design of Jovin, et al. (31), by Andrew E. Seer Jr., Master Glassblower, Department of Chemistry, Michigan State University, East Lansing, Michigan. An important design modification was made consisting of a u-tube which was designed to hold the lower buffer and the anode. This simplified the operation of the device since it did not have to be partially immersed in a large jar containing the lower buffer. The electrophoresis unit was operated in a 5 C cold room and was cooled by circulating ice water. At the current loads used for this study, the gel temperature was maintained below 5 C.

The gel system was a modification of that reported by Ornstein and Davis (68). The stock solutions used for preparation of the gels contained the following components taken to a final volume of 100 ml with distilled water:

A	B
4.0 ml conc. HCl	4.0 ml conc. HCl
0.23 ml TEMED	0.46 ml TEMED
36.3 g Tris	5.98 g Tris
Final pH, 8.8 to 8.9	Final pH, 7.3 to 7.4

CN	DN
30.0 g acrylamide	14.0 g acrylamide
0.8 g bisacrylamide	0.25 g bisacrylamide

E	Catalyst
4.0 mg riboflavin	0.14 g ammonium persulfate

The lower separating gel contained the following amounts of the stock solutions in a final volume of 100 ml;

Parts	Stock
1	A
2	CN
4	Catal.

These were mixed, placed in the column, overlaid with distilled water, and allowed to polymerize at 5 C for 2 hr.

The upper stacking gel contained the following proportions of stock solutions in a final volume of 50 ml;

Parts	Stock
1	B
2	DN
1	E
4	H_2O

The water was removed from the top of the polymerized lower gel and the upper gel mixture was added. The stacking gel was photopolymerized for 2 hr at 5 C with four 15 watt fluorescent lights.

The upper buffer consisted of 5.76 g glycine and 1.2 g Tris in a final volume of 2 liters, pH 8.3. The lower/elution buffer was made by adding solid Tris to 3 liters of distilled water containing 15 ml of conc. HCl, until a pH of 8.1 was obtained. A freshly prepared gel column was used for each electrophoresis run.

Analytical Polyacrylamide Disc-gel Electrophoresis

The analytical disc-gel electrophoresis technique of Ornstein and Davis (68) was used to determine the purity of a variety of PNPase preparations. The electrophoresis buffer contained 28.8 g glycine and 6.0 g Tris per liter with a final pH of 8.3. This buffer was diluted 10-fold with distilled water before use. The stock solutions used for preparation of the gels contained the following components taken to a final volume of 100 ml with distilled water:

A	B
4.0 ml conc. HCl	2.8 ml 1 N HCl
0.23 ml TEMED	0.45 ml TEMED
36.6 g Tris	5.98 g Tris
Final pH 8.9	Final pH 6.7

C	D
20.0 g acrylamide	10.0 g acrylamide
0.735 g bisacrylamide	2.5 g bisacrylamide

E	Catalyst
4.0 mg riboflavin	0.14 g ammonium persulfate

F
40.0 g sucrose

The lower separating gel contained the following proportions of the stock solutions;

Parts	Stock
1	A
2	C
4	Catal.
1	H_2O

These were mixed and 0.7 ml were placed in each 5 x 75 mm electrophoresis tube. The gels were overlaid with 0.1 ml of distilled water and allowed to polymerize for 40 min at 25 C.

The upper spacer gel contained the following proportions of the stock solutions;

Parts	Stock
1	B
2	D
1	E
4	F

These were combined and 0.15 ml of the mixture was placed over the polymerized lower gel after the water layer had been removed. The upper gel was photopolymerized under a layer of distilled water with four 15 watt fluorescent lights for 10 min at 25 C. The gels were "pre-electrophoresed" at 5 C for 30 min at 2 ma per tube.

Protein (5 µg to 100 µg per tube) was introduced in 100 µl of an 8% sucrose solution. Approximately 0.2 ml of a 0.01% brom phenol blue solution in distilled water was mixed into the upper buffer reservoir to serve as an anionic marker. Electrophoresis was carried out at 5 C with a constant current of 1.5 ma/tube until the dye marker had migrated half-way through the upper gel. The current was then increased to 2 ma/tube and maintained at that level for the remainder of the run. The gels were removed from their respective tubes and a 3 mm length of fine copper wire was inserted horizontally through each gel to mark the position of the dye marker. This was necessary because subsequent procedures led to loss of the marker. Duplicate gels were run for each protein sample. One gel was stained for protein and the other for PNPase activity.

The gels to be stained for protein were immersed for 10 min in 1% buffalo black dye in 7% acetic acid. The gels were destained by standing for 48 hr in 7% acetic acid.

The gels to be stained for PNPase activity were washed with distilled water, placed in 14 x 100 mm test tubes and covered with a solution consisting of 0.3 ml of 50 mM Tris-HCl; 0.1 ml of 10mM inosine; and 0.1 ml of 50 mM sodium arsenate. This combination was incubated at 37 C for 15 min. The gels were then removed and washed well with distilled water. Each gel was covered with 5 ml of 1 N NaOH and 1 ml of 0.5% triphenyltetrazolium chloride solution and placed in a boiling water bath for a few minutes until a red precipitate started to form. Then they were washed with distilled water and stored in 7% acetic acid.

Kinetic Studies of Vegetative Cell and Spore PNPase

A standard Lineweaver-Burk treatment of the Michaelis-Menten equation was used in this study. The Michaelis-Menten equation for a single substrate is:

$$v = \frac{V_{max}\ (S)}{K_m + (S)}$$

where v, is the initial velocity; V_{max}, is the velocity obtained under saturating substrate (S) conditions; and K_m, is the Michaelis constant (the concentration of substrate which produces half of the maximal velocity). This equation was rearranged by Lineweaver and Burk (53) to give:

$$\frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{1}{(S)} + \frac{1}{V_{max}}$$

which was plotted as 1/v vs. 1/(S).

The reaction mechanism of PNPase is bimolecular since two substrates were involved (inosine and phosphate). Actual kinetic analysis indicated that a "ping-pong" or shuttle mechanism (10) was not involved.

Cleland (11) has shown that the following rate equation will describe the initial velocity for a bi-reactant, sequential mechanism (where both substrates must be present on the enzyme surface before the reaction occurs) in the presence of all reactants:

$$v = \frac{V_1 \, A \, B}{K_{ia} \, K_b + K_b \, A + K_a \, B + AB}$$

where it is assumed that inosine (A) combines with the enzyme before the phosphate (B) is added. This assumption was based on the findings of Kim, et al. (38) who

studied the kinetics of human erythrocyte PNPase and found that inosine did bind first. The terms are defined as follows:

A = inosine concentration, Molar.

B = phosphate concentration, Molar.

K_a = Michaelis constant for A, B saturating.

K_b = Michaelis constant for B, A saturating.

K_{ia} = dissociation constant for EA complex (E=enzyme).

v = initial velocity, units per minute.

V_1 = maximal velocity, A and B saturating, in the forward direction.

V_1/E_t = turnover number, units per mg protein.

Pure vegetative cell and spore enzymes were assayed at varying concentrations of both substrates. Standard Lineweaver-Burk plots were constructed for each enzyme with either phosphate as the changing fixed substrate and inosine as the variable substrate or with inosine as the changing fixed substrate and phosphate as the variable substrate.[1]

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[1]Notation used by Kim, et al. (38) where the concentration of the second substrate was held constant and the concentration of the first substrate was varied to produce one line on the Lineweaver-Burk plot. Another concentration of the second substrate was chosen and held constant while the concentration of the first substrate was varied to produce a different line on the Lineweaver-Burk plot and so on until a family of lines were produced.

The family of intercepts from each Lineweaver-Burk plot were replotted against the reciprocal of the changing fixed substrate concentration. When the resulting line was extrapolated to the vertical axis, a value of V_1 for that particular Lineweaver-Burk plot was obtained. The reasoning for this procedure was as follows.

When kinetic data are plotted on a standard 1/v vs. 1/substrate plot, the intercept of the line at the vertical axis is the miximal velocity obtainable at saturation of the variable substrate in the presence of the fixed amount of the second substrate used. When the amount of the second substrate is changed, different maximal velocities (intercepts) are obtained. Different maximal velocities will be found until saturating amounts of the second substrate are used, after which the intercept will no longer change. The replot of the intercepts vs. the reciprocal of the changing fixed substrate when extrapolated to the vertical axis, gives the maximal velocity obtained at saturating concentrations of both inosine and phosphate.

The family of slopes from each Lineweaver-Burk plot were replotted against the reciprocal of the changing fixed substrate concentration. When the resulting line was extrapolated to the vertical axis, a value for the Michaelis constant divided by the maximal velocity was obtained. When this value was multiplied by the V_1

value found from the previous replot, a Michaelis constant for the variable substrate being studied was found. The reasoning for this procedure was as follows.

When kinetic data are plotted according to the Lineweaver-Burk relationship, the slope of the line is the Michaelis constant for the variable substrate divided by a particular maximal velocity. That maximal velocity represents the value obtained at saturation of the variable substrate in the presence of the fixed amount of the second substrate used. When the amount of the second substrate is changed, different Michaelis constants and maximal velocities will be obtained. The slope will continue to change until saturating amounts of the second substrate are used. In this case, the slope will no longer change since the enzyme is at maximal velocity. The replot of the slopes vs. the reciprocal of the changing fixed substrate, when extrapolated to the vertical axis, gives the Michaelis constant divided by the maximal velocity under saturation conditions of both substrates. When this value is multiplied by the maximal velocity found in the presence of saturating substrates (V_1) the true Michaelis constant for the variable substrate is found. This constant is independent of the changing fixed substrate concentration.

An alternative method was also used for finding the Michaelis constant for the variable substrate. The

line obtained from assays near saturating levels of the changing fixed substrate was extrapolated to the horizontal axis on the Lineweaver-Burk plot. This intercept was equal to the reciprocal of the Michaelis constant. The procedure is less precise than the above method since extrapolation is less accurate at high concentrations of the changing fixed substrate.

Sucrose Density Gradient Centrifugation

Analytical grade sucrose was used to make linear 5 to 20% gradients by the method of Martin and Ames (59). The 4.55 ml gradients were made at 5 C in 1.27 x 5.08 cm cellulose nitrate tubes (Beckman-Spinco, Palo Alto, Calif.). The gradients were kept at this temperature for 3 hr before use. Each gradient was layered with equal parts of lactic acid dehydrogenase (LDH, 0.10 mg/ml) and PNPase (0.25 mg/ml) in a total volume of 100 µl. The gradients were subjected to centrifugation in a Spinco Model L preparative ultracentrifuge (Beckman-Spinco, Palo Alto, Calif.) with a SW-39L rotor at 36,000 rpm for 20 hr at 5 C. Immediately after centrifugation, 37 to 38 3-drop fractions were collected from each gradient and assayed for LDH and PNPase activity. The LDH was assayed according to the procedure presented by Worthington Biochemical Corporation (98).

Protein Absorption Spectrum of Spore PNPase

Protein spectra were measured in the standard buffer with a Shimadzu double beam diffraction grating spectrophotometer (Seisakusho Limited, Kyoto, Japan) incorporating an expanded absorbance scale meter. Absorbance readings were made in 1 nm wavelength increments from 290 to 340 nm with a 1 nm bandwidth. Expanded scale absorbance readings were made in 1 nm increments from 250 to 290 nm with a 0.5 nm bandwidth. Molar absorptivity was calculated using the standard Beer's law relationship (9):

$$A \equiv E = -\log_{10} T = e\ l\ c$$

where A, is the absorbance; E, is the extinction; T, is the transmittance; e, is the molar absorptivity; c, is the concentration in moles per liter; and l, is the length of the light path in cm.

Subunit Analysis by SDS Polyacrylamide Gel Electrophoresis

Subunit analysis of PNPase was performed by a modification of the method reported by Shapiro, et al. (81). The subunit molecular weights of proteins which have been disaggregated in sodium lauryl sulfate (SDS) are proportional to their migration rates in acrylamide gels. The stock solutions used to make the 5.0%

acrylamide gels contained the following components taken to a final volume of 100 ml with distilled water:

A
8.0 ml, 1 M sodium phosphate buffer, pH 7.1 0.23 ml TEMED

C	G
20.0 g acrylamide 0.735 g bisacrylamide	0.14 g ammonium persulfate

The stock solutions were combined in the following proportions;

Parts	Stock
1	A
2	C
4	G
1	H_2O

To each of 12, 5 x 75 mm tubes was added 1.5 ml of gel mixture. The gel was overlaid with distilled water and polymerized at 25 C for 30 min.

The electrophoresis buffer contained 0.1% SDS in 100 mM sodium phosphate buffer, pH 7.0. The gels were "pre-electrophoresed" at 7 ma per tube for 30 min at 25 C.

The protein dissociation mixture contained equal proportions of the following components which were combined just before use: 10% SDS in distilled water;

10% (v/v) 2-mercaptoethanol in distilled water; and 100 mM sodium phosphate buffer, pH 6.8.

The marker mixture contained 0.01% brom phenol blue, 10 mM dithiothreitol, and 50% (v/v) glycerol in 10 mM sodium phosphate buffer, pH 7.0. DNase I, RNase, and bovine serum albumin (1 mg/ml stock solutions) were used as subunit molecular weight markers. Their molecular weights after dissociation in SDS were 31,000 (52), 13,700 (96, 99), and 68,000 (87, 91) respectively.

The protein stain was made by dissolving 250 mg of coomassie brilliant blue in 10 ml of distilled water. To this solution was added 5 ml of methanol and enough 10% trichloroacetic acid (TCA) in distilled water to bring the total volume to 100 ml. The solution was mixed well and kept overnight. Just before use, the stain was filtered through Whatman #1 filter paper. After the gels had been fixed in 10% TCA, they were placed in 14 x 100 mm tubes and covered with the stain solution. The staining procedure took 10 hr at 25 C, after which time the gels were destained by standing in 10% TCA for 48 hr.

High Speed Sedimentation Equilibrium Molecular Weight Study

A Spinco analytical Model E ultracentrifuge (Beckman-Spinco, Palo Alto, Calif.) equipped with phase-plate interference optics was used for this study. An AN-D rotor was used with standard double sector cells whose

solution column depths were 3 mm (110 µl of sample per cell column). Techniques were available to allow multicell operation with interference optics. The PNPase samples were subjected to centrifugation at 17,100 rpm for 24 hr at 4.5 C. Simultaneous photographs of the interference patterns for each cell were taken. The fringe patterns, which are a measure of protein concentration differences within each cell, were measured with a Bausch and Lomb two-dimensional comparator. The photographic plate was aligned on the comparator X-coordinate and the displacement along the most defined fringe was measured along the Y-coordinate. Measurements were taken at intervals of 50 µ on the X-coordinate. A partial specific volume of 0.717 ml/gm, obtained from an amino acid analysis of vegetative cell PNPase (16), was assigned for both the vegetative cell and spore PNPase enzymes. A pycnometer (29) was used to determine the density of the standard buffer (1.00022 g/cc) and that of the standard buffer plus 10 mM potassium phosphate (1.00163 g/cc) at 20 C.

Complete statistical analysis of the data by the method of Yphantis (100) was performed using a computer program which was adapted to a Control Data Corporation 3600 digital computer by workers in the Department of Biochemistry, Michigan State University, East Lansing, Michigan. A discussion of this analysis is presented in

Appendix B. The data obtained from the computer printout are summarized below:

Whole-cell average, weight-average molecular weight.

Number-average molecular weight at zero concentration (Mn), from the extrapolated slope of a molecular weight vs. protein concentration plot.

Weight-average molecular weight at zero concentration (Mw), from the extrapolated slope of a molecular weight vs. protein concentration plot.

Plots of the weight-average molecular weight vs. protein concentration.

Plots of the number-average molecular weight vs. protein concentration.

Plots of the ln of fringe displacement (protein concentration) vs. the squared distance from the center of rotation.

All of these data were statistically analyzed and presented with accompanying confidence intervals.

The fundamental relationship between the molecular weight and sedimentation coefficient for a spherical protein under ideal conditions can be solved. By assigning the value 0.717 ml/gm for the partial specific volume of PNPase, the following equation yields the sedimentation coefficient for a hypothetical spherical PNPase molecule with the experimentally determined molecular weight:

$$S^{o} = 3.79 \times 10^{-3} M^{2/3}$$

where S^{o}, is the sedimentation coefficient in Svedberg units; and M, is the molecular weight (Appendix C). With this equation and the whole cell weight-average

molecular weight values obtained from the high speed sedimentation equilibrium study, sedimentation coefficients were calculated for both the vegetative cell and spore PNPase enzymes in the presence or absence of 10 mM potassium phosphate.

Using equation I from Appendix C, minimal frictional coefficients (f_o) were calculated from the molecular weight data and calculated S^o values. The actual frictional coefficients (f) were also calculated from the molecular weight data and the experimentally obtained sedimentation data. From the f/f_o ratios an indication of the molecular asymmetry could be determined. A perfectly spherical molecule would have a f/f_o ratio of 1.0. Globular proteins have ratios in the range of 1.1 to 2.0.

RESULTS

Purification of Purine Nucleoside Phosphorylase

The PNPase from vegetative cells and spores of B. cereus T was purified so that each enzyme could be studied in the absence of uncontrolled interactions between it and other cell components. The purification procedure was a modification of those used previously (16, 20).

All purification steps were carried out at 5 C. Centrifugation was at 20,000 x g for 60 min. Both the vegetative cell and spore PNPase enzymes were purified by the same procedures except that the spores were heat treated before they were broken. All dialysis tubing was boiled 1 min in 10 μM ethylenediaminetetraacetate (EDTA) to remove trace metals before use.

Preparation of Crude Extract

Vegetative cells (500 g, wet weight) were suspended in 1.2 liters of the standard buffer and washed into an Eppenbach colloid mill. To the mill were added 1 kg of #110 glass beads and 3 ml of antifoam B. The cells were ground at full speed for 40 min at 10 C. This procedure resulted in over 80% cell breakage as judged by phase contrast microscopy. The mixture was removed from the

mill and the extract was separated from the beads. The cell extract and 300 ml of buffer used to wash the glass beads were then clarified by centrifugation. The supernate was filtered through cheesecloth and assayed. This was designated fraction I.

Washed spores (360 g, wet weight) were suspended in 800 ml of the standard buffer and heated to 70 C for 30 min to inactivate any contaminating vegetative cell enzyme. The spores remained refractile under phase contrast microscopy after this heat treatment. The spores were quickly cooled in a -20 C alcohol bath and washed into the colloid mill along with an additional 400 ml of buffer. The spores were broken at full speed in the presence of 1 kg of #110 glass beads and 3 ml of antifoam B for 55 min at 10 C. Less than 15% of the cells remained intact after this procedure. The spore extract was combined with 200 ml of buffer used to wash the beads and clarified by centrifugation. The supernatant fluid was filtered through cheesecloth and assayed as spore PNPase, fraction I.

Approximately 4-times more enzyme was recovered from vegetative cells than from spores, based on equal starting wet weights of cells and spores.

Streptomycin Sulfate Precipitation

Nucleic acids were partially removed from the crude extracts by slowly adding 3.3 g of streptomycin sulfate

for each liter of extract. The suspensions were stirred for an additional 60 min and then allowed to settle at 5 C for 12 hr. The precipitate was removed by centrifugation and the supernate containing the PNPase activity was filtered through cheesecloth to remove the lipid-like material which did not sediment.

Ammonium Sulfate Fractionation of Crude Extract

The enzyme solution from the streptomycin step was taken to 50% saturation by slowly adding solid ammonium sulfate (288 g/liter) with stirring in an ice bath. The solution was stirred for an additional 60 min and then clarified by centrifugation. The precipitated protein contained very little PNPase activity after dialysis and was discarded. The supernatant was taken to 80% saturation with an additional 197 g/liter of solid ammonium sulfate, stirred for 60 min, and the protein precipitate was removed by centrifugation. The pellet containing the PNPase activity was resuspended in the standard buffer and dialyzed against 4 liters of the same buffer for 16 hr. The enzyme was stored at -15 C with no appreciable loss of activity. This preparation was designated fraction II.

DEAE-cellulose Column Chromatography

After several unproductive purification attempts were made using gel filtration methods, it was discovered

that DEAE-cellulose chromatography was a more efficient process. Various combinations of pH and ionic strength were tested but the following procedure was the most effective.

Spore PNPase fraction II was thawed and 67 ml (837.5 mg of protein) were placed on a DEAE-cellulose column (3.5 x 100 cm) which had been equilibrated with 2 liters of 240 mM Tris-HCl, 10 mM 2-mercaptoethanol buffer, pH 7.5. The protein was eluted from the column with 2.5 liters of the above buffer and the column effluent was collected in 21 ml fractions (25 min per fraction). This zero-gradient elution removed most of the protein from the column. A linear gradient was then started consisting of 1.5 liters of 240 mM Tris-HCl, 10 mM 2-mercaptoethanol buffer, pH 7.5, and 1.5 liters of 330 mM Tris-HCl, 10 mM 2-mercaptoethanol buffer, pH 7.5. The PNPase activity eluted after 1.6 liters of eluent had passed through the column. This corresponded to elution in 290 mM Tris. Ninety percent of the enzyme placed on the column was recovered with a 21-fold increase in specific activity for the peak fractions. This preparation was called spore fraction III.

The vegetative cell PNPase fraction II was thawed and 110 ml (1.375 mg of protein) were placed on a 3.5 x 100 cm DEAE-cellulose column and eluted with 240 mM Tris-HCl, 10 mM 2-mercaptoethanol buffer, pH 7.5.

Eighteen-milliliter fractions were collected (30 min per fraction). After 3.6 liters of buffer had passed through the column, a 3 liter linear gradient was started as before but no further enzyme activity was eluted off. The recovery was 87% of the enzyme activity. A 6-fold increase in specific activity was obtained for the peak fractions after they were pooled and concentrated. This preparation was designated fraction III.

Vegetative cell PNPase differed from the spore enzyme by eluting at lower Tris concentrations. This may have been due to a difference in size and/or charge between the two enzymes.

Preparative Disc-gel Electrophoresis

The preparative disc-gel method had been previously used in this laboratory to purify PNPase (16). The total recovery of enzyme from this procedure was usually only 50% when a modification of the gel system reported by Jovin, et al. (31) was used. Further modifications of the separating gels during this investigation resulted in yields of up to 90%.

Spore PNPase fraction III was made 8% (w/v) with respect to sucrose and 100 µl of a 0.01% solution of brom phenol blue in distilled water was added for anionic marker. The mixture was layered over the preparative polyacrylamide gel and a constant current of 15 ma was

applied to the column. The ammonium persulfate was eluted from the column with buffer at 0.25 ml/min. When the dye marker had migrated half-way through the upper stacking gel, the current was increased to 20 ma and kept at this level for the remainder of the run. As the marker entered the lower separating gel, the elution rate was increased to 0.5 ml/min. When the marker had moved to the lower extremity of the column, the elution rate was increased to 1.5 ml/min and fractions were collected every 10 min. The fraction containing the marker was designated tube 1.

As shown in Figure 1, the PNPase activity came off the column with very little accompanying 280 nm absorbing material. Tubes 32 through 35 were pooled and concentrated. Eighty percent of the enzyme placed on the column was recovered with a 13-fold increase in specific activity for the peak fractions.

Fraction III of the vegetative cell enzyme was placed on a similar electrophoresis column and run under identical conditions. The final elution rate was 1.2 ml/min. As shown in Figure 2, the activity was eluted from the column without a measurable 280 nm absorbing peak. Tubes 28 and 29 were combined and concentrated by ultrafiltration. Over a 90% recovery of enzyme activity was found with a 38-fold increase in specific activity for the peak fractions.

Figure 1. Elution profile of spore purine nucleoside phosphorylase from the preparative disc-gel electrophoresis column. Current was maintained at 20 ma. The elution rate was 1.5 ml/min and the fraction volumes were 15 ml. The ordinates are activity (u=units ojenyyme) and absorbance. See text for experimental details.

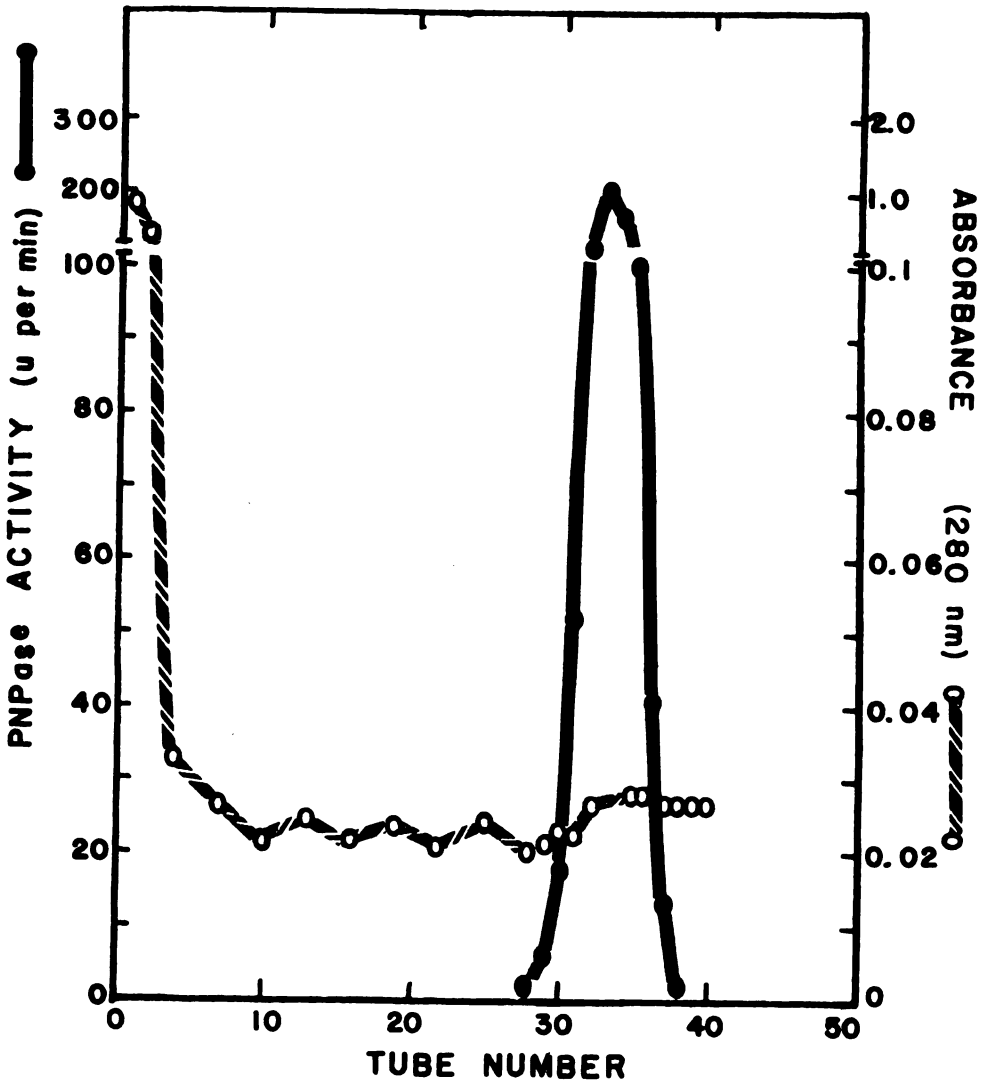
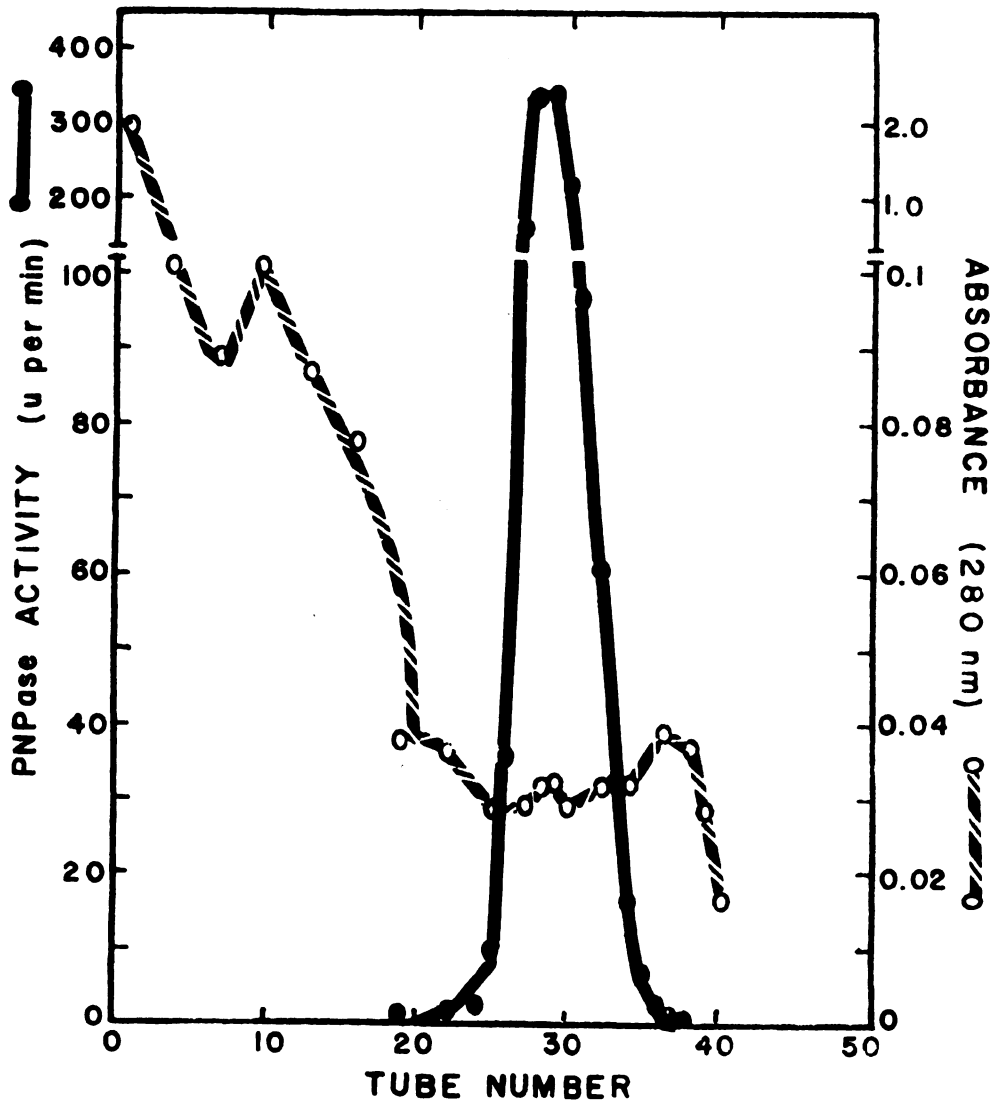

PNPase ACTIVITY (u per min)
300
200
100
80
60
40
20
0
0
10
20
30
40
50
TUBE NUMBER
2.0
1.0
0.1
0.08
0.06
0.04
0.02
0
ABSORBANCE (280 nm)

Figure 2. Elution profile of vegetative cell purine nucleoside phosphorylase from the preparative disc-gel electrophoresis column. Current was maintained at 20 ma. The elution rate was 1.2 ml/min and the fraction volumes were 12 ml. The ordinates are activity (u=units of enzyme) and absorbance. Experimental details are given in the text.


PNPase ACTIVITY (u per min)
ABSORBANCE (280 nm)
TUBE NUMBER
0
20
40
60
80
100
200
300
400
0.02
0.04
0.06
0.08
0.1
1.0
2.0
10
30
50

The protein concentrations for all of the pure PNPase preparations were below the measurable range of assay by the Lowry method. The enzymes were concentrated by putting them into small dialysis bags, covering them with dry Sephadex G-25 and dehydrating for 12 hr at 5 C. The dialysis bags containing the concentrated PNPases were then dialyzed against the standard buffer and stored at -15 C. These preparations were called fractions IV.

The rapid migration of this enzyme under the conditions used in this procedure made the purification of relatively large quantities of enzyme possible. A summary of the overall purification procedures for the vegetative cell and spore PNPases are presented in Tables 1 and 2 respectively.

Thermal Inactivation of Purine Nucleoside Phosphorylase

A previous study indicated that the spore PNPase was stable but became thermal labile in the presence of phosphate ion (16). An effort was made to determine the basis for the effect of phosphate on heat resistance.

Two-milliliter samples of spore and vegetative cell PNPase from the DEAE-cellulose column (fractions III) were each dialyzed for 24 hr against 200 ml of the standard buffer at 5 C. An enzyme solution (200 µl) was diluted with 200 µl of the standard buffer and placed in a 14 x 100 mm test tube. After a zero time assay sample

TABLE 1.--Purification of vegetative cell purine nucleoside phosphorylase.

	Fraction	Total Enzyme Activity	Specific Activity	Purification	Recovery
		units	units/mg	fold	%
I.	Crude extract	171,000	7.4	1	100
II.	Streptomycin sulfate + ammonium sulfate (50-80%), dialyzed	132,800	46.3	6	78
III.	DEAE-cellulose column[a]	72,400	293.0	40	42
IV.	Prep. disc-gel electrophoresis[a]	39,100	8,600.0	1,160	23

[a]Only the pooled peak fractions are represented. Corrected for having used only part of fraction II. Experimental details are reported in the text.

TABLE 2.--Purification of spore purine nucleoside phosphorylase.

	Fraction	Total Enzyme Activity	Specific Activity	Purification	Recovery
		units	units/mg	fold	%
I.	Crude extract	30,400	1.1	1	100
II.	Streptomycin sulfate + ammonium sulfate (50-80%), dialyzed	26,500	26.5	24	87
III.	DEAE-cellulose column[a]	17,100	570.0	518	56
IV.	Prep. disc-gel electrophoresis[a]	11,400	7,670.0	6,970	37

[a]Only the pooled peak fractions are represented. Corrected for having used only part of fraction II. See text for experimental details.

was withdrawn, the tube was immersed in a 60 C water bath and assayed after 2, 5, and 10 min for remaining PNPase activity. As shown in Figure 3, both the vegetative cell and spore-derived enzymes were heat labile under these conditions (half-life of 0.5 min).

When 100 µl of the same enzyme solutions were diluted with 100 µl of the standard buffer containing 100 mM potassium phosphate at pH 7.5 (final phosphate concentration of 50 mM) both PNPase preparations were stabilized about 48-fold (half-life of 24 min). Neither enzyme was stabilized in the presence of 10 mM inosine or by phosphate concentrations below 10 mM. Electrophoretically homogeneous vegetative cell and spore PNPase samples showed similar denaturation characteristics.

Thermal inactivation experiments were conducted with crude vegetative cell and spore extracts containing PNPase activity (fractions I). At 60 C in the standard buffer, their respective half-lives were 32 min and 1.5 min. When the vegetative cell and spore enzymes were tested in the standard buffer containing 50 mM potassium phosphate, their respective half-lives increased to 225 min and 70 min. The PNPase from the vegetative cell crude extract was more thermal stable than the crude spore enzyme.

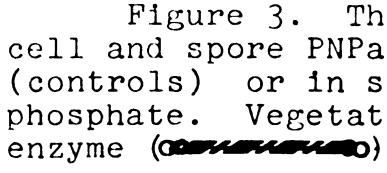
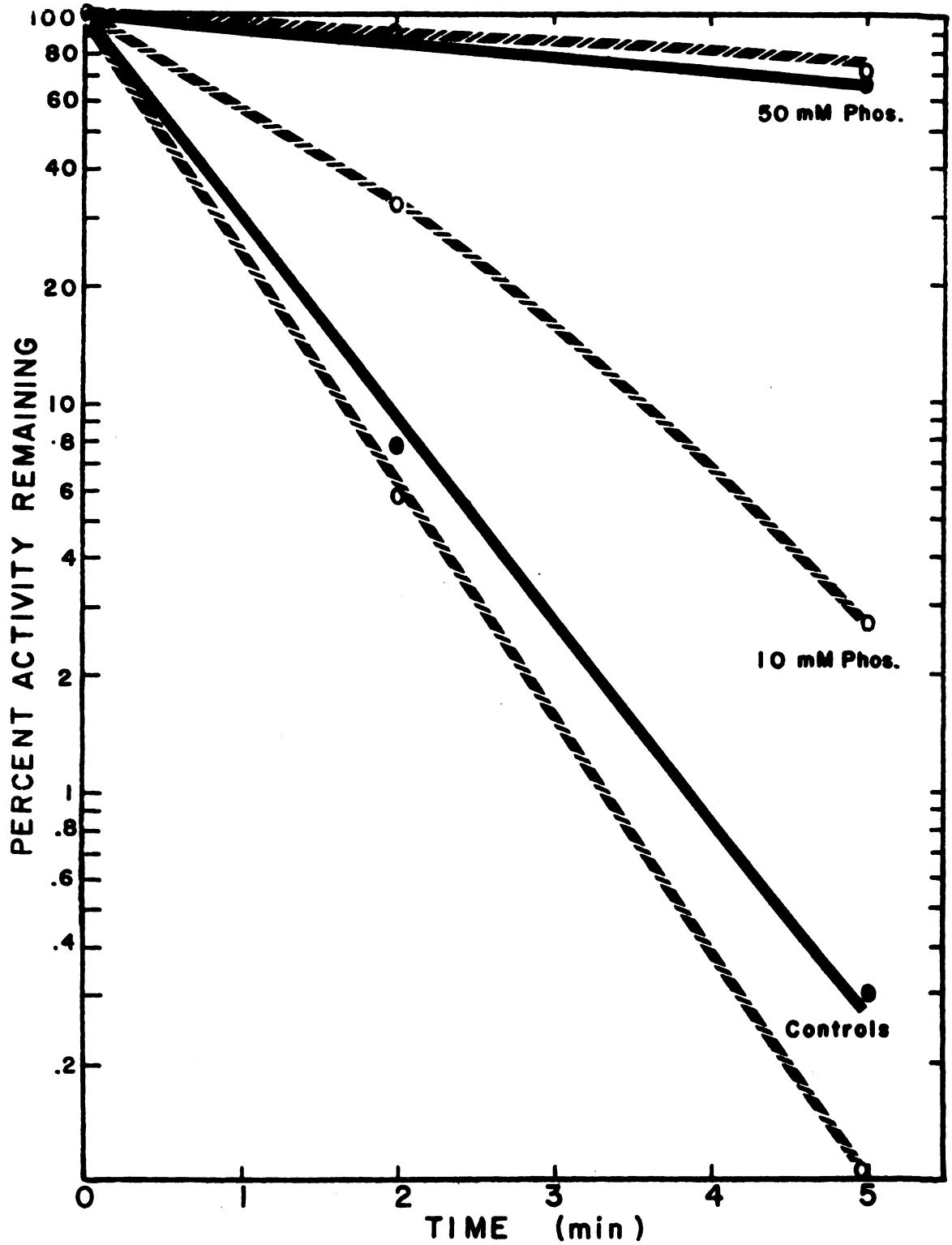


Figure 3. Thermal inactivation of vegetative cell and spore PNPase at 60 C in standard buffer (controls) or in standard buffer containing potassium phosphate. Vegetative cell enzyme (●——●); spore enzyme (○——○). See text for experimental details.


100
80
60
40
20
10
8
6
4
2
1
.8
.6
.4
.2
PERCENT ACTIVITY REMAINING
50 mM Phos.
10 mM Phos.
Controls
0
1
2
3
4
5
TIME (min)

Samples of vegetative cell and spore PNPase (fractions I) were then dialyzed in the standard buffer containing 10 mM $CaCl_2$. The half-lives of the vegetative cell and spore enzymes were 10 min and 3.0 min respectively. The calcium ion labilized the vegetative cell PNPase and appeared to stabilize the spore enzyme. When 20 mM potassium phosphate was added to these preparations, the vegetative cell PNPase increased to a half-life of 30 min and the spore PNPase increased to a half-life of 10 min. Therefore, the addition of phosphate produced an increase in heat stability for both enzyme preparations in either the presence or absence of calcium ion.

Analytical Polyacrylamide Disc-gel Electrophoresis of PNPase

Samples of purified vegetative cell and spore PNPase (fractions IV) were checked for electrophoretic homogeneity using the analytical disc-gel electrophoresis technique. Both the vegetative cell and spore PNPase enzymes from the preparative polyacrylamide electrophoresis columns behaved in an identical manner. A single protein-staining band which migrated with the PNPase activity was found for either enzyme indicating that each preparation was electrophoretically homogeneous. The mobilities of the vegetative cell and spore enzymes were similar. The results of a typical run are shown in Figure 4.

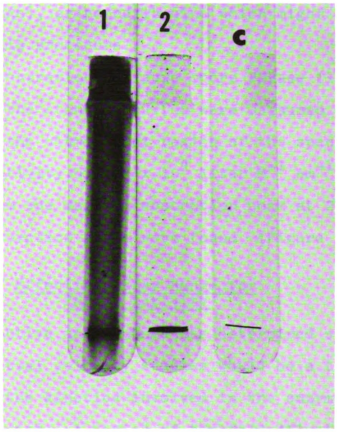


Figure 4.--Analytical polyacrylamide disc-gel electrophoresis of spore PNPase. Tube #1, was stained for PNPase activity. Tube #2, was stained for protein. The control tube (C), contained no protein and was a control on marker migration. A control gel without protein (not shown) did not produce a band when stained for PNPase activity. See text for the experimental procedures.

Ultraviolet Absorption Spectrum of Pure Spore PNPase

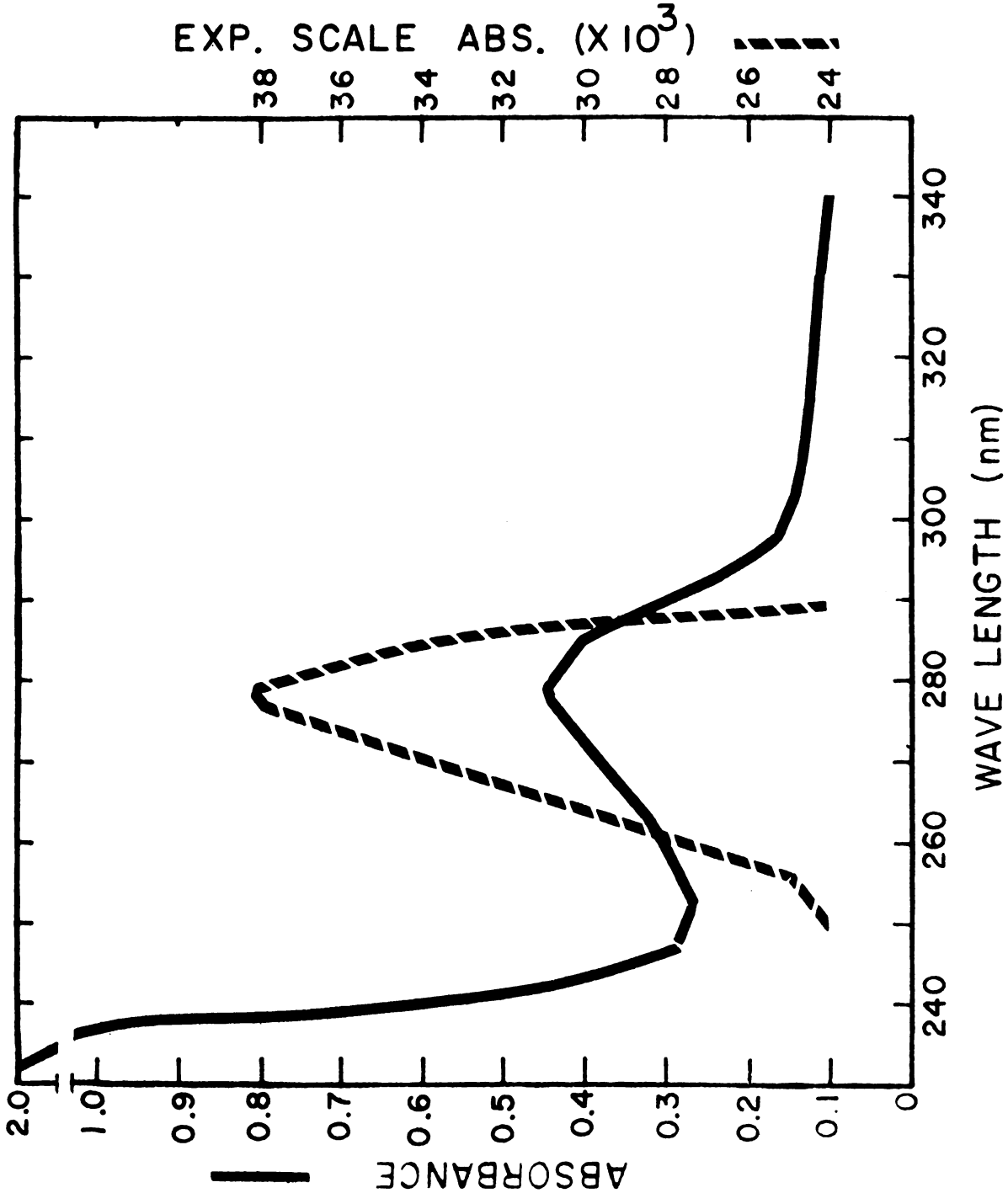
An absorption spectrum of pure spore PNPase was measured to determine whether the protein contained bound cofactors, unique amino acid structures (viz. thiazoline rings), or bound inosine which might be detected by this procedure.

Two concentrations of pure spore PNPase (142 and 700 µg/ml) in the standard buffer were measured for absorption in the ultraviolet region of the spectrum. A molar absorptivity of 4.7×10^4 liter M^{-1} cm^{-1} was calculated from the maximum absorption peak at 279 nm. No unusual absorption peaks were found (Figure 5).

Effective Assay Range for PNPase Activity

The effective assay range was determined so that subsequent initial velocity studies would be a true reflection of the kinetics involved in the enzyme reaction mechanism. A series of ten-fold dilutions of pure spore and vegetative cell PNPase were made in the standard buffer. Aliquots (100 µl) were assayed in the standard assay system at 37 C. The optimum enzyme content per assay for both enzymes ranged from 0.2 to 2.0 µg of protein and no lag in initial velocity was noted.

Figure 5. Ultraviolet absorption spectrum of pure spore PNPase in the standard buffer where the absorbance is plotted vs. wave length. Also included is an expanded scale plot over the absorbance range of 24 - 38 x 10 $^{-3}$. See text for the experimental details.


EXP. SCALE ABS. (X 10³)
38
36
34
32
30
28
26
24
2.0
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE
240
260
280
300
320
340
WAVE LENGTH (nm)

Subunit Activity of Spore PNPase

A previous study indicated that in the absence of phosphate ion (16) the spore PNPase was smaller than the vegetative cell enzyme. An effort was made to determine whether a concentration-dependent aggregation of protein was a necessary prerequisite for spore PNPase catalytic activity.

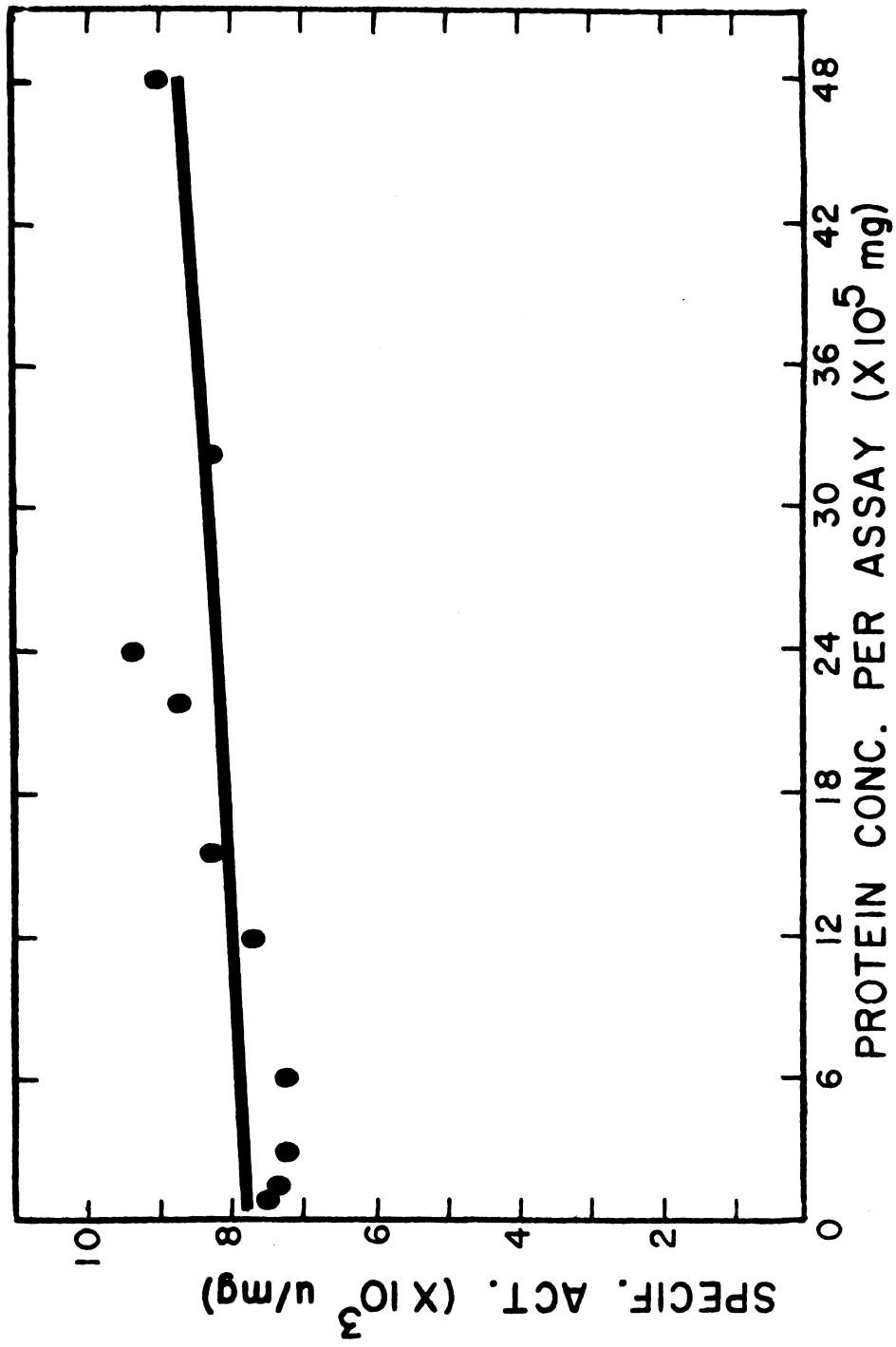
Pure spore PNPase (fraction IV) was assayed at various protein concentrations in the standard assay system. The calculated specific activity was then plotted against the protein concentration used for each assay. As shown by Figure 6, the slope was essentially zero over a broad concentration range.

Initial Velocity Analysis of Vegetative Cell and Spore PNPase

Kim, et al. (37, 38) studied initial velocities and reported unusual kinetics for the PNPase from human erythrocytes. A previous study of vegetative cell and spore PNPase in this laboratory also indicated non-linear Lineweaver-Burk plots (16). The kinetics of both enzymes were therefore studied at various concentrations of each substrate in order to ascertain the extent of the catalytic differences between the two proteins.

The pure vegetative cell and spore enzymes (fractions IV) were diluted in the standard buffer to 0.95

Figure 6. Subunit activity of spore PNPase. Purified enzyme (fraction IV) was assayed at various protein concentrations in the standard assay system and the calculated specific activity (u=units) was plotted against the protein concentration used for each assay. See text for details.


SPECIF. ACT. (X 10^3 u/mg)
10
8
6
4
2
0
6
12
18
24
30
36
42
48
PROTEIN CONC. PER ASSAY (X 10^5 mg)

and 1.15 µg/ml respectively. All dilutions of the phosphate buffer and inosine stock solutions were made in the standard buffer. All kinetic data were collected using the same enzyme dilutions and reagents on the same day in order to minimize variations in the results. All assays were performed at 37 C after the assay mixtures had been equilibrated for 10 min in the cuvette changer. The reactions were started by adding the enzyme.

With phosphate as the changing fixed substrate and inosine as the variable substrate, the Lineweaver-Burk plots (Figures 7 and 8) were linear for both the vegetative cell and spore enzymes. Since the plots intersected to the left of the vertical axis, a sequential reaction mechanism was indicated (11). Using the terminology of Cleland (10) for a bisubstrate reaction, the average Michaelis constants for inosine (K_a) were 4.6 x 10^{-4} M and 7.0 x 10^{-4} M for the vegetative cell and spore PNPase enzymes respectively. There was a 1.5-fold difference in the K_a values for the two enzymes.

With inosine as the changing fixed substrate and phosphate as the variable substrate, the Lineweaver-Burk plots (Figures 9 and 10) showed a downward curvature at low inosine concentrations. Kim, et al. (37, 38) described a similar downward curvature for the PNPase from human erythrocytes. When the intercepts were replotted vs. the reciprocal of the inosine concentration, the

Figure 7. Initial velocity of vegetative cell PNPase with inosine as the variable substrate. Plot of reciprocal of velocity vs. the reciprocal of inosine concentrations over a range of phosphate concentrations. The kinetic parameter estimated from this plot and replots (<u>inset</u>) is: K_a, 4.6×10^{-4} M. Experimental details are reported in the text.

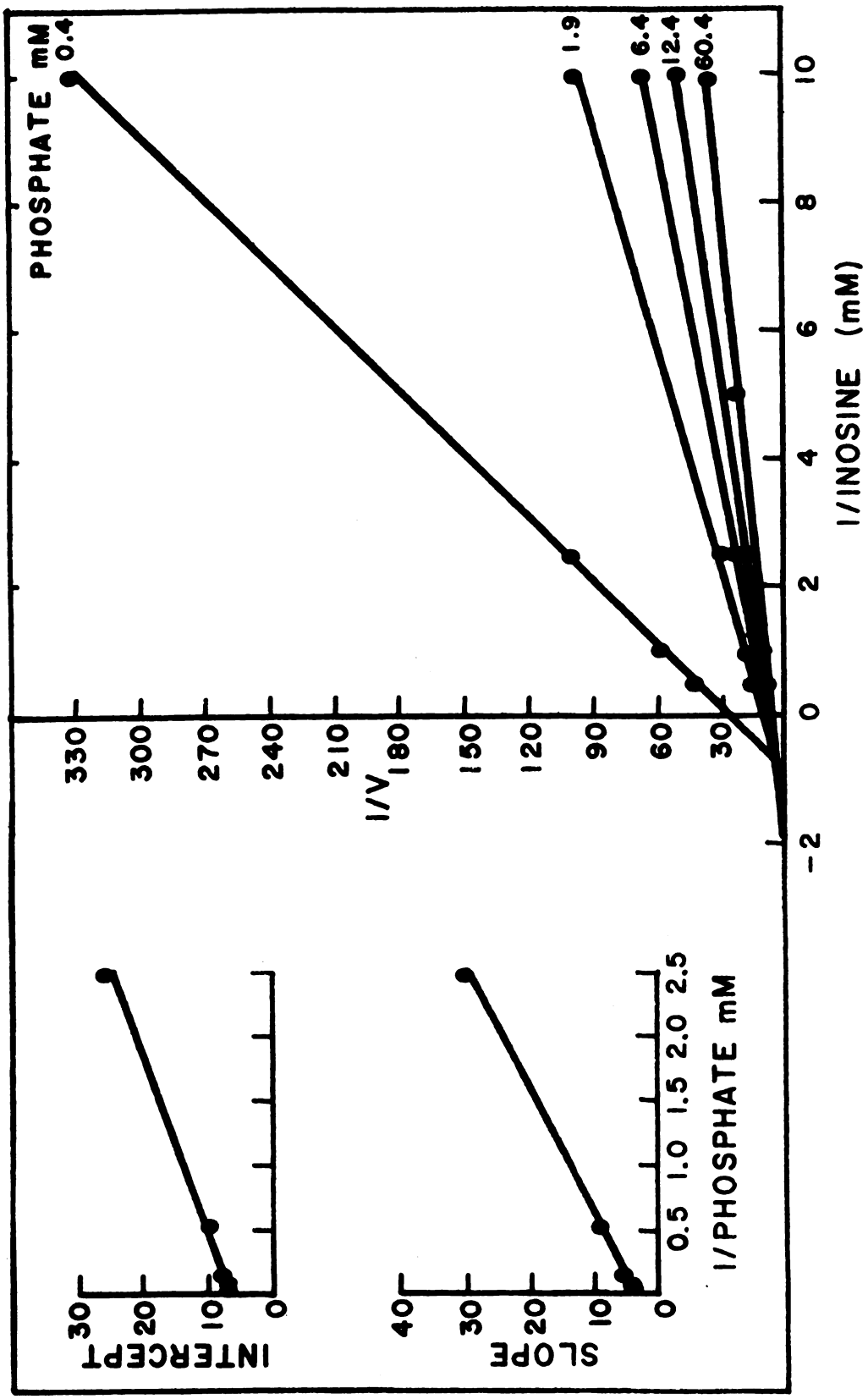

PHOSPHATE mM
0.4
1.9
6.4
12.4
60.4
1/V
330
300
270
240
210
180
150
120
90
60
30
-2
0
2
4
6
8
10
1/INOSINE (mM)
INTERCEPT
30
20
10
0
SLOPE
40
30
20
10
0
0.5 1.0 1.5 2.0 2.5
1/PHOSPHATE mM

Figure 8. Initial velocity of spore PNPase with inosine as the variable substrate. Plot of reciprocal of velocity vs. the reciprocal of inosine concentrations over a range of phosphate concentrations. The kinetic parameter estimated from this plot and replots (<u>inset</u>) is: K_a, 7.0×10^{-4} M. See text for experimental details.

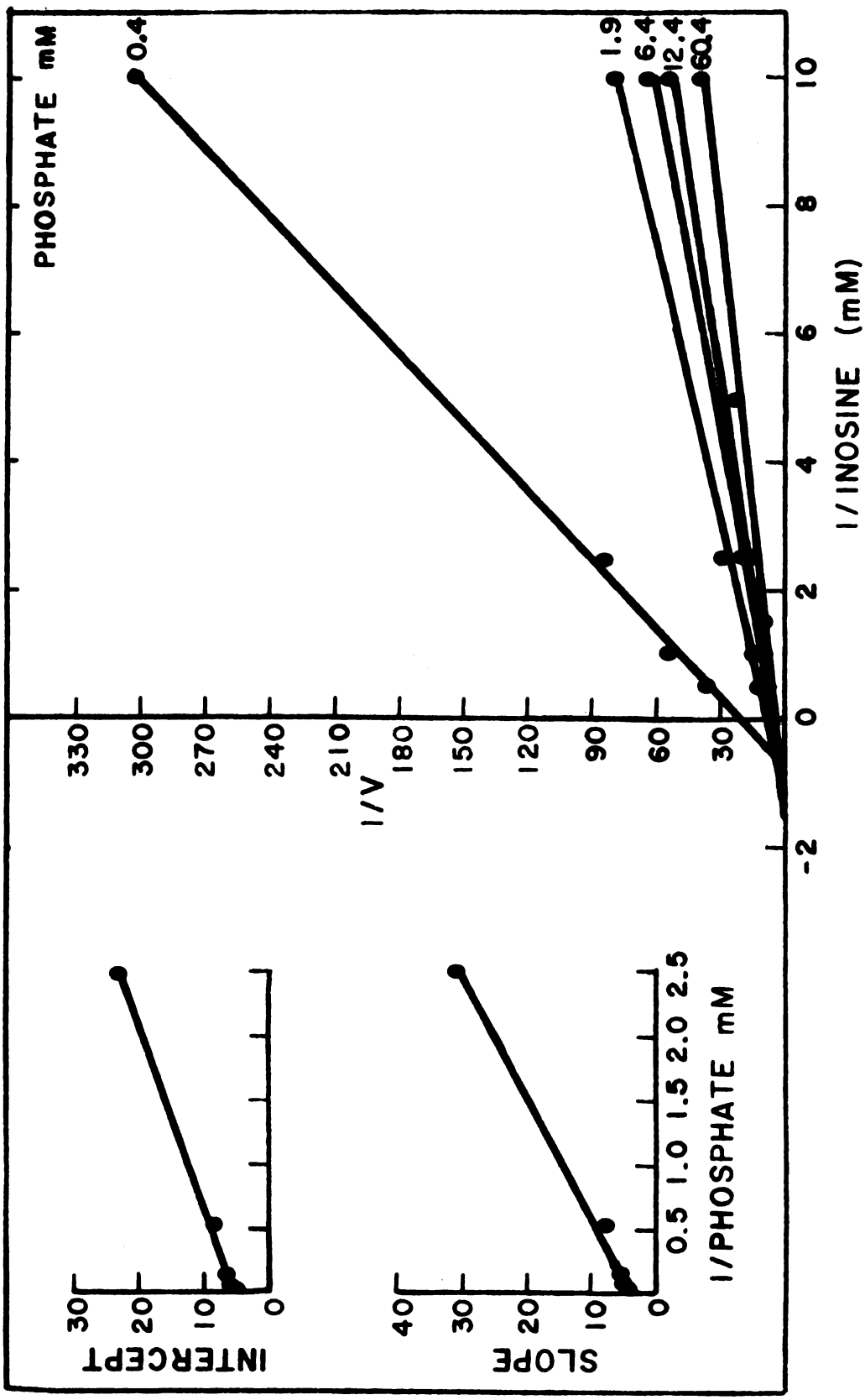

PHOSPHATE mM
0.4
1.9
6.4
12.4
60.4
1/V
330
300
270
240
210
180
150
120
90
60
30
-2
0
2
4
6
8
10
1/INOSINE (mM)
INTERCEPT
30
20
10
0
SLOPE
40
30
20
10
0
0.5 1.0 1.5 2.0 2.5
1/PHOSPHATE mM

Figure 9. Initial velocity of vegetative cell PNPase with phosphate as the variable substrate. Plot of reciprocal of velocity vs. the reciprocal of phosphate concentrations over a range of inosine concentrations. The kinetic parameter estimated from this plot and replots (inset) is: K_b, 1.5 x 10^{-3} M. See the text for experimental procedures.

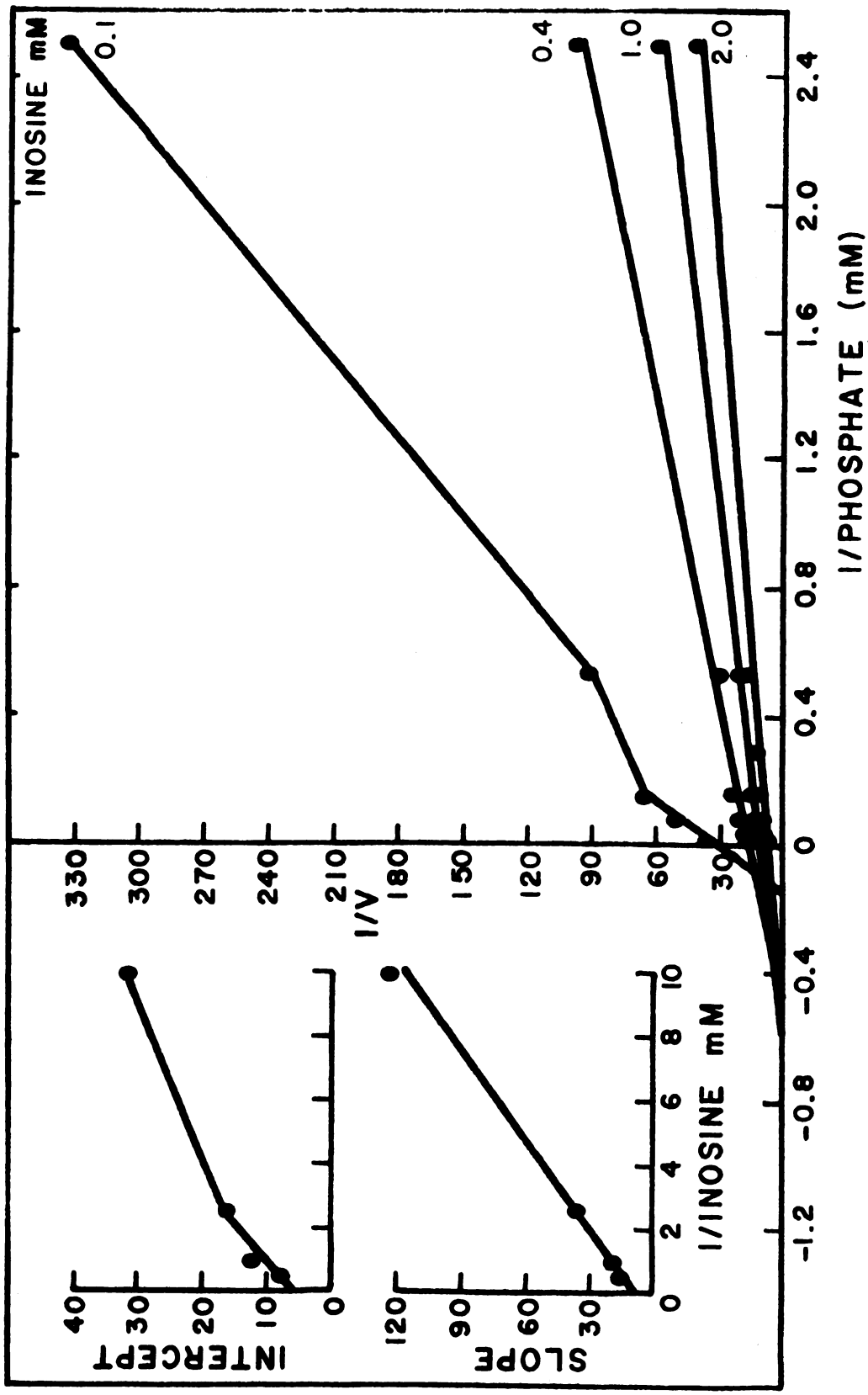
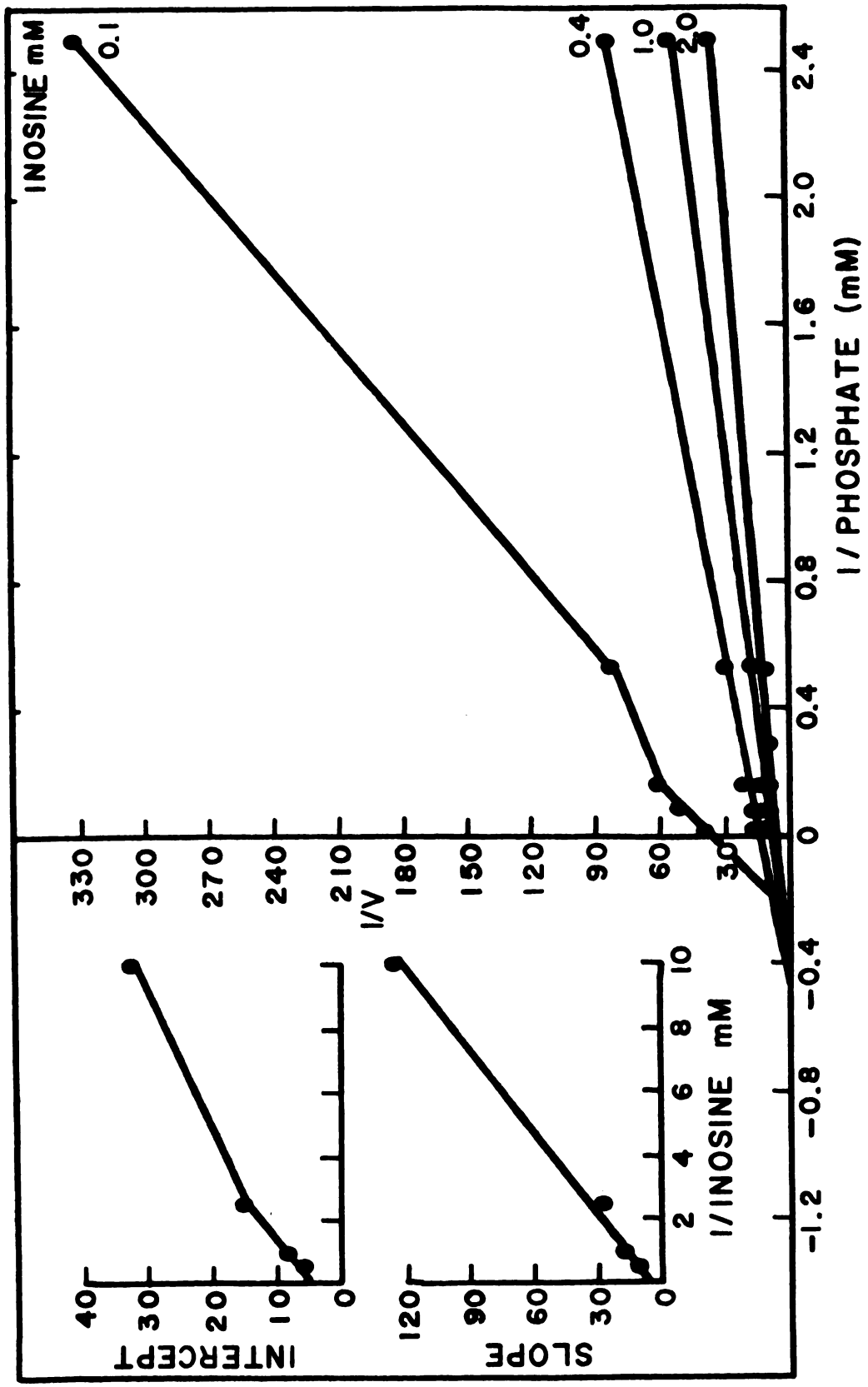

INOSINE mM
0.1
0.4
1.0
2.0
1/V
330
300
270
240
210
180
150
120
90
60
30
1/PHOSPHATE (mM)
-1.2
-0.8
-0.4
0
0.4
0.8
1.2
1.6
2.0
2.4
INTERCEPT
40
30
20
10
0
SLOPE
120
90
60
30
0
2
4
6
8
10
1/INOSINE mM

Figure 10. Initial velocity of spore PNPase with phosphate as the variable substrate. Plot of reciprocal of velocity vs. the reciprocal of phosphate concentrations over a range of inosine concentrations. The kinetic parameter estimated from this plot and replots (inset) is: K_b, 1.3×10^{-3} M. Experimental details are reported in the text.


INOSINE mM
0.1
0.4
1.0
2.0
1/V
1/ PHOSPHATE (mM)
INTERCEPT
SLOPE
1/INOSINE mM

results were not linear. This may have been due to either subunit interaction (30) or to negative cooperative effects between the two substrates (15). The Michaelis constants for phosphate (K_b) were 1.5 x 10^{-3} M and 1.3 x 10^{-3} M for the vegetative cell and spore PNPases respectively.

The turnover numbers (V_1/E_t) for the vegetative cell and spore enzymes at saturating levels of both substrates were 8,320 and 8,690 units/mg protein respectively. A summary of the kinetic data is presented in Table 3.

Subunit Size of Purine Nucleoside Phosphorylase

Although cell and spore PNPases appear to be synthesized under the direction of the same genomic unit (20), a previous study indicated that the ratio between their molecular weights was unusual (16). This phenomenon could have been the result of a proteolytic conversion of vegetative cell to spore PNPase where a portion of the vegetative enzyme was lost. A precedent for this mechanism existed in the conversion of vegetative to spore aldolase in B. cereus (77). This kind of proteolysis might lead to an unequal modification of subunits. Therefore, an investigation was made of the vegetative cell and spore enzyme subunit sizes.

TABLE 3.--Michaelis constants of the substrates for pure vegetative cell and spore PNPases.

Parameter[a]	Spore PNPase	Veg. PNPase
Inosine, K_a	7.0×10^{-4} M	4.6×10^{-4} M
Phosphate, K_b	1.3×10^{-3} M	1.5×10^{-3} M
Turnover number[b]	8,690 units/mg	8,320 units/mg

[a]Average Michaelis constant values are given from the replots of the slopes and intercepts of the Lineweaver-Burk plots (Figures 7-10) and from the direct plots.

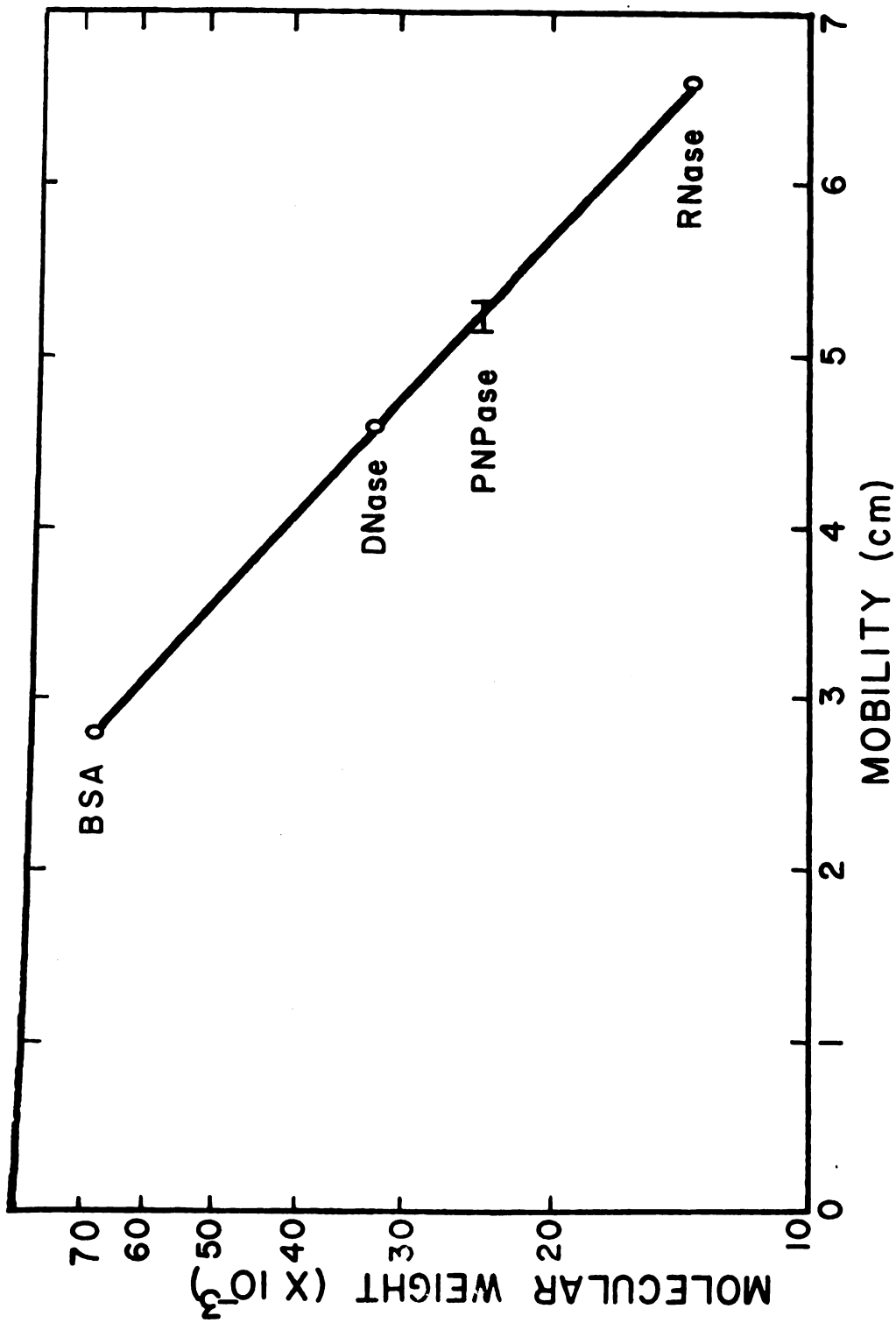
[b]The turnover numbers were calculated from (V_1/E_t); where E_t, was the concentration of enzyme (mg) in the assay and V_1, was the units of enzyme activity/min in the assay. Experimental details are given in the text.

Duplicate samples of pure vegetative cell PNPase (100 µl, 0.38 mg/ml), spore PNPase (100 µl, 0.46 mg/ml), and three marker proteins (100 µl, 1.0 mg/ml) were each added to separate 6 x 50 mm tubes. To each tube was added 50 µl of the dissociation mixture and the samples were incubated for 3 hr at 37 C. A marker-glycerol mixture (20 µl) was added to each tube and 10 to 30 µg of protein was introduced onto the top of polyacrylamide gels. The proteins were "electrophoresed" at 7 ma/tube for 30 min at 25 C. The gels were fixed in 10% TCA, stained and decolorized. The protein band migration distance was measured from the top of each gel. A plot of the log of molecular weight vs. migration distance was linear (Figure 11) and the subunit size for both the vegetative cell and spore PNPases was 24,000 ±10%. This experiment was repeated with a current of 4 ma/tube and the same results were obtained.

Sucrose Density Gradient Sedimentation of PNPase

The sedimentation properties of spore PNPase are affected by phosphate ion (16). This behavior was investigated at varying phosphate concentrations to determine whether subunit association could be detected at high phosphate concentrations. It was also of interest to determine whether the phosphate effect was reversible.

Figure 11. Determination of the molecular weight of the polypeptide chains of vegetative cell and spore PNPases by SDS-polyacrylamide gel electrophoresis. The three marker proteins used were bovine serum albumin, deoxyribonuclease I, and ribonuclease. Experimental procedures are presented in the text.


BSA
DNase
PNPase
RNase
MOLECULAR WEIGHT (X 10³)
MOBILITY (cm)
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7

Pure spore and vegetative enzymes (fractions IV) were diluted in standard buffer to a concentration of 0.25 mg/ml and dialyzed for 24 hr at 5 C. Each sucrose gradient was layered with 100 µl of enzyme solution containing 125 µg of either vegetative cell or spore PNPase combined with 50 µg of LDH marker. The gradients were spun at 36,000 rpm for 20 hr at 5 C. In the standard buffer system the sedimentation velocities for the vegetative cell and spore enzymes were 5.5 S and 5.3 S $\pm$ 0.1 S respectively. The sedimentation coefficients of the PNPase preparations were calculated by assigning a sedimentation value of 7.6 S to the LDH marker (36, 62). Studies using LDH in sucrose density gradients employing buffer systems similar to those used in this investigation indicated that its sedimentation coefficient did not change in the presence of phosphate ion (George M. Stancel, Department of Biochemistry, Michigan State University, East Lansing, Michigan; personal communication).

The peak PNPase activity fractions were pooled, concentrated and dialyzed for 18 hr at 5 C against the standard buffer containing 1 mM KH_2PO_4, at pH 7.5. The enzyme solutions were mixed with the marker enzyme, layered on sucrose gradients prepared in the same buffer system and sedimented as before. The vegetative cell enzyme had a sedimentation coefficient of 5.5 S and the

spore PNPase had a value of 5.4 S $\pm$ 0.1 S. The vegetative cell enzyme sedimentation velocity did not change but the value for the spore enzyme increased (Table 4).

In another study, pure vegetative cell and spore PNPase solutions were diluted to give a protein concentration of 0.25 mg/ml. Three aliquots of each enzyme were then dialyzed against the standard buffer for 24 hr at 5 C. Two aliquots of each enzyme were then dialyzed against the above buffer containing 10 mM KH_2PO_4, pH 7.5 for 24 hr. At this time, one aliquot of each enzyme was returned to the standard Tris buffer, while the other aliquot remained in the Tris + phosphate buffer. Dialysis was continued for an additional 48 hr with several changes of buffer. The enzymes were then sedimented through sucrose gradients containing the same buffer in which they were finally dialyzed. The results are tabulated in Table 4.

A study was made to determine whether there was a protein concentration-dependent change in sedimentation velocity for the spore PNPase in the standard buffer. A pure spore PNPase preparation (fraction IV) was dialyzed against the standard buffer and three different concentrations (40 µg, 150 µg, and 230 µg) were each mixed with the LDH marker and layered on separate sucrose gradients. The enzymes were sedimented through the gradients at 36,000 rpm for 24 hr at 5 C. The PNPase activities

TABLE 4.--Sucrose density gradient centrifugation of pure vegetative cell and spore PNPases.

Buffer[a]	Sedimentation coefficients	
	Spore PNPase	Veg. PNPase
	$s_{20,w}$ I0.1 s	$s_{20,w}$ I0.1 s
Standard buffer	5.3	5.5
Standard buffer + 1 mM Phos.	5.4	5.5
Standard buffer + 10 mM phos. then standard buffer	5.6	5.5
Standard buffer + 10 mM Phos.	5.7	5.5

[a]The standard buffer contained 50 mM Tris-HCl, 10 mM 2-mercaptoethanol, pH 7.5. The other buffers had a pH of 7.5. See text for the experimental procedures.

sedimented at the same rate relative to the LDH marker regardless of the PNPase protein concentration. The calculated sedimentation velocities ranged between 5.3 S and 5.4 S ± 0.1 S. Therefore, no protein aggregation occurred within the range of protein concentrations tested.

High Speed Sedimentation Equilibrium Molecular Weights

Accurate molecular weight determinations for both the vegetative cell and spore enzymes were needed to exploit the findings of previous investigators (16, 20). These data could then be used to calculate frictional coefficients, substantiate the homogeneity of the enzyme preparations, and to demonstrate subunit association in the presence of phosphate.

Both the vegetative cell and spore enzymes (fractions IV) were dialyzed and subjected to centrifugation in the standard Tris buffer or in that buffer containing 10 mM potassium phosphate. A previous sedimentation velocity study in the Model E ultracentrifuge indicated that the spore enzyme did not undergo aggregation in the Tris buffer system. The enzymes were placed in separate double sector cells and spun at 17,100 rpm for 24 hr at 4.5 C. Simultaneous photographs of the interference patterns for each cell were then taken. All of the molecular weight data were collected from the same

experiment so that external variables were more controlled. The results given in Table 5 include a complete statistical analysis at the 99% confidence level.

The plots of the natural logarithm of the fringe displacement (ln D) vs. the square of the distance from the center of rotation (r^2) were linear except near the top of the sector cells containing the spore enzyme (Figures 12 and 13). The enzyme preparations appeared to be homogeneous. The limiting slopes near the top and bottom of the sector cells as well as the overall slopes are tabulated in Table 5.

The size of the vegetative cell PNPase did not change in the presence or absence of 10 mM phosphate. The spore enzyme was smaller in the Tris buffer and appeared to increase to a size similar to that of the vegetative cell enzyme in the presence of phosphate.

The f/f_o ratios (for calculations see Appendix C) indicated that the spore enzyme was quite spherical in Tris buffer and then assumed the shape of a more globular protein in the presence of phosphate (Table 6). This indicated that the molecular weight increase of the spore PNPase in the presence of phosphate ion probably produced a change in shape as well as a change in size.

TABLE 5.--Molecular weights of vegetative cell and spore PNPase by sedimentation equilibrium.

Enzyme-buffer[a]	Whole-cell average molecular weight	Weight-average molecular weight, c = 0	Number-average molecular weight, c = 0	Slope of ln D vs. r^2		
				Top	Bottom	Overall
Spore PNPase						
Tris	69,900 ± 500	61,300 ± 2,300	53,800 ± 500	1.225	1.469	1.370
Tris + Phos.	94,800 ± 600	102,000 ± 3,200	97,800 ± 600	2.084	1.732	1.870
Veg. PNPase						
Tris	91,600 ± 400	94,100 ± 3,000	91,300 ± 500	1.834	1.834	1.834
Tris + Phos.	88,300 ± 400	95,900 ± 3,000	93,700 ± 500	1.757	1.757	1.757

[a]Pure vegetative cell and spore PNPase enzymes were prepared and studied under the conditions given in the text. The (Tris) buffer contained 50 mM Tris-HCl, 10 mM 2-mercaptoethanol, pH 7.5; and the (Tris + Phos.) buffer contained 50 mM Tris-HCl, 10 mM 2-mercaptoethanol, 10 mM potassium phosphate, pH 7.5.

Figure 12. Plots of ln D vs. r^2 for spore PNPase. Enzyme in standard buffer (o——————o). Enzyme in standard buffer + 10 mM potassium phosphate (●——————●). See Appendix B and text for experimental procedures.

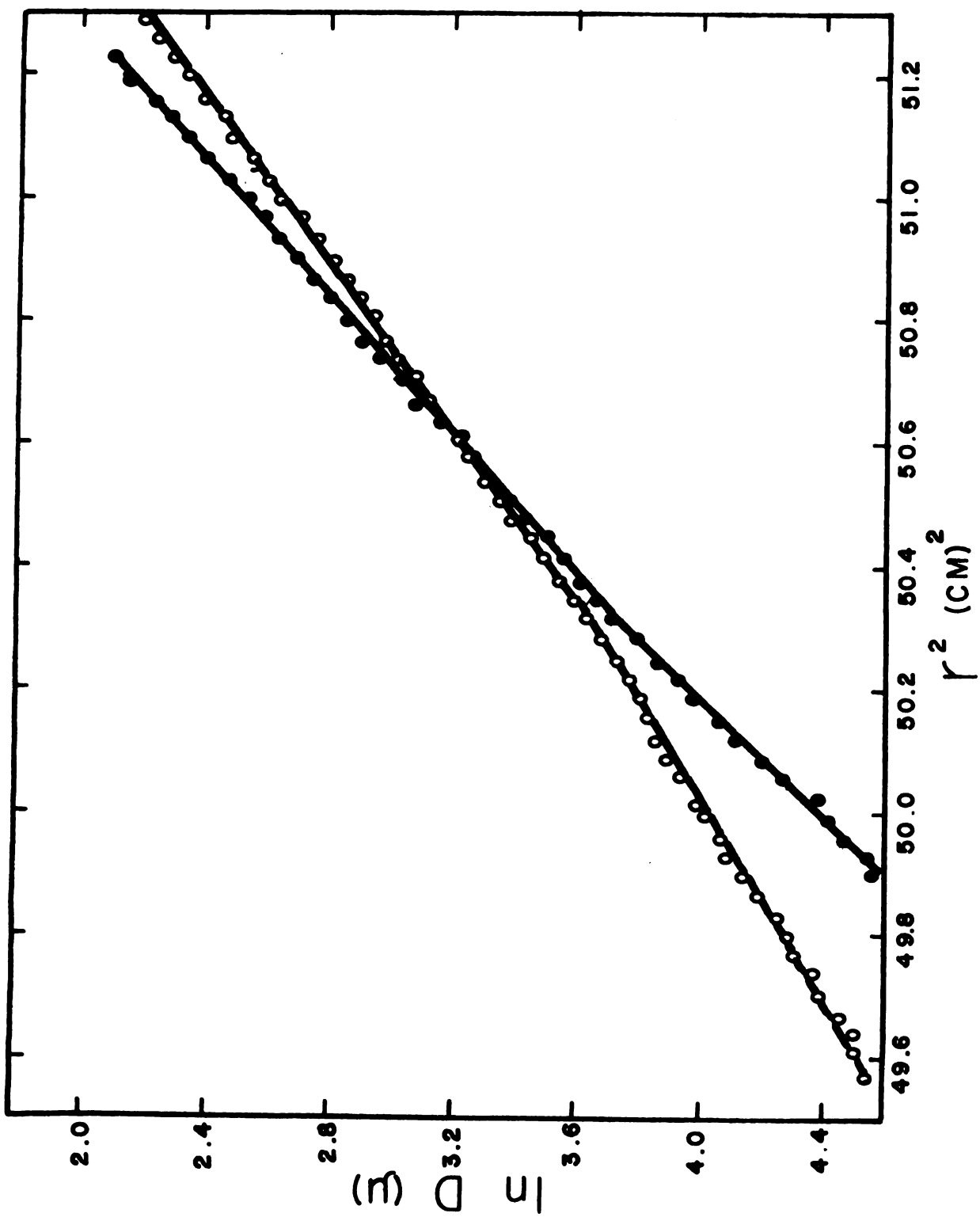

ln D (w)
2.0
2.4
2.8
3.2
3.6
4.0
4.4
49.6
49.8
50.0
50.2
50.4
50.6
50.8
51.0
51.2
r² (CM)²

Figure 13. Plots of ln D vs. r^2 for vegetative cell PNPase. Enzyme in standard buffer (o——————o). Enzyme in standard buffer + 10 mM potassium phosphate (●——————●). See Appendix B. Experimental details are presented in the text.

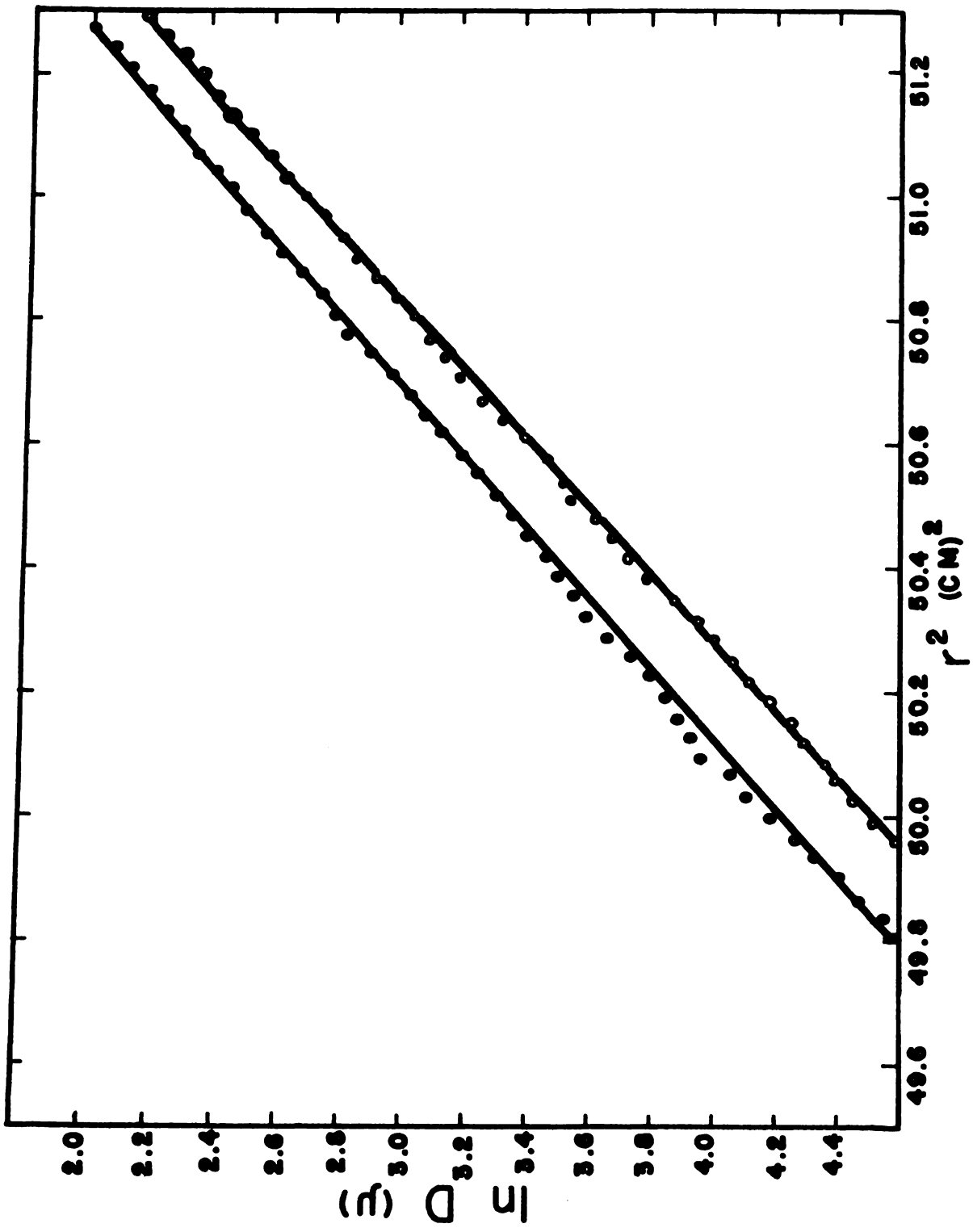

ln D (μ)
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6
3.8
4.0
4.2
4.4
49.6
49.8
50.0
50.2
50.4
50.6
50.8
51.0
51.2
r² (CM)²

TABLE 6.--Calculated frictional ratios of vegetative cells and spore PNPases.

Enzyme-buffer[a]	Whole-cell Molecular Weight	$s_{20,w}$ ±0.1s	f/f_0[b]
Spore PNPase			
Tris	69,900	5.3	1.2
Tris + 10 mM P	94,800	5.7	1.4
Vegetative PNPase			
Tris	91,600	5.5	1.4
Tris + 10 mM P	88,300	5.5	1.4

[a]Tris buffer contained 50 mM Tris-HCl, 10 mM 2-mercaptoethanol, pH 7.5. Tris + 10 mM P buffer contained the above components plus 10 mM potassium phosphate, pH 7.5.

[b]The value for f_o was calculated from the whole-cell average molecular weight, based on the sedimentation coefficient which a protein of this weight would have if it were spherical (Appendix B). See text for experimental details.

DISCUSSION

The cell and spore PNPases had been purified by slightly different procedures in previous studies (16, 20). In this investigation both enzymes were purified by the same procedure in order to minimize any effects which might be due to methods of enzyme preparation. The only significant difference in the purification of the two enzymes was the heat treatment of the spores before they were broken. It was assumed that the PNPase protein within the spores would be protected from thermal denaturation whereas the vegetative cell PNPase would be inactivated. The heat treatment did not seem to initiate germination since no loss in refractility was detected when the spores were examined by phase microscopy. The vegetative cell-derived crude extract had approximately 4-times more PNPase activity than the spore extract, based on equal starting weights. A preliminary study indicated that the level of PNPase activity increased in vegetative cells toward the end of exponential growth and remained at this level in the spores. The lower yield of spore PNPase activity found in this investigation may be the result of protein denaturation during the mechanical breaking of the

spores. Spores are particularly resistant to rupture and require a more prolonged grinding than do vegetative cells. Alternatively, the heating may have denatured some of the spore enzyme.

Engelbrecht and Sadoff (16) reported that the spore enzyme was more stable than the vegetative enzyme in the absence of phosphate and possessed the same low thermal stability as the vegetative enzyme in the presence of phosphate. This was not found with the enzyme preparations used in this study.

Both of the partially purified vegetative cell and spore PNPase proteins were labile in the absence of phosphate at 60 C. The enzymes used in the previous investigation were not electrophoretically homogeneous preparations and may have contained some unknown stabilizer which was stripped from the enzymes used in this work during the DEAE-cellulose purification step. This possibility was supported by the thermal inactivation studies employing crude extracts of vegetative cells and spores in which the vegetative cell crude extract contained PNPase which was more stable than the extract containing the spore enzyme. After both preparations were purified by DEAE-cellulose chromatography their thermal stabilities were similar.

The differential effect of phosphate ion on the thermal stability of cell and spore PNPase was also found

to be different from that reported previously (16). The presence of phosphate ion stabilized both the vegetative cell and spore PNPase proteins from either crude extracts or from the electrophoretically homogeneous preparations. The reason for this difference was not immediately apparent. The spore enzyme previously studied may have possessed some heat-stabilizing ion which was subsequently removed in the presence of phosphate.

The enzymes used in this investigation were routinely kept in a sulfhydral-protective environment (10 mM 2-mercaptoethanol) whereas the preparations used by Engelbrecht and Sadoff (16) were not. The thermal stability of spore PNPase from crude extract was not affected by the presence or absence of 2-mercaptoethanol since similar half-lives were obtained at 60 C when the enzyme was tested in either 50 mM imidiazole buffer, pH 7.5 or in the standard buffer. The maintenance of sulfhydral bonds during the purification of these enzymes could have affected their thermal stabilities. The absence of a sulfhydral protective reagent could have resulted in oxidation and the formation of some disulfide bridges which would permit a conformational change within the spore enzyme so that it could become more heat stable (7).

The spore PNPase preparation which was used in the previous thermal inactivation study (16) was eluted from

a calcium phosphate gel column before it was dialyzed and tested for thermal stability. It is possible that this enzyme preparation contained residual amounts of calcium ion which could have affected its stability. Vegetative cell and spore PNPases from crude extracts were tested in the presence of 10 mM $CaCl_2$. The spore enzyme was slightly stabilized and the vegetative cell enzyme became labile.

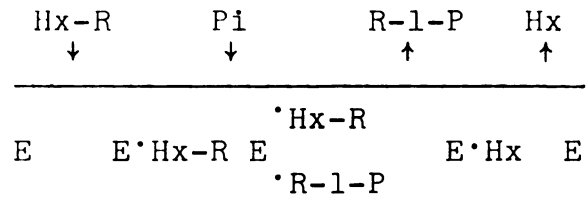
Heating the spores to 70 C for 30 min could have resulted in their activation and subsequent germination had the proper agents been present in the buffer. Germination materials could have been derived from residual material remaining after the spores were washed. If some germination of the spore population did occur, the extracted PNPase could have possessed a lowered heat stability. However, the spores were all refractile before they were placed in the colloid mill as were the remaining spores after the cell grinding procedure. The denaturation of any vegetative cell PNPase present in the spore crop was more important to the goals of this study (separation of cell and spore PNPases) than the possibility of activation effects.

No unusual components were detected in the ultraviolet absorbance spectrum of spore PNPase. Therefore, the differences in molecular weight between the spore and vegetative cell PNPase enzymes previously reported

(16) were not due to the binding or release of a cofactor or other easily characterized ultraviolet-absorbing component.

The spore enzyme appeared to be catalytically active in its lowest molecular weight configuration. The plot of specific activity vs. protein concentration was linear and essentially had a zero slope. Had a protein concentration-dependent aggregation been necessary for activity, the plot would have shown a drop in specific activity at the low protein concentrations. It is possible that the presence of phosphate in the assay mixtures could have promoted aggregation of PNPase even at low protein concentrations.

The kinetic analysis indicated a complicated reaction mechanism. The data suggest that an ordered, sequential reaction occurs since the Lineweaver-Burk plots for phosphate as the changing fixed substrate and inosine as the variable substrate (or reverse) intersected to the left of the vertical axis (11). In this reaction mechanism, the first substrate, inosine, binds to the enzyme and is followed by the second substrate, phosphate. This reaction mechanism was proposed by Kim, et al. (38) from inhibition studies with the PNPase derived from human erythrocytes. The reaction mechanism is graphically represented by;



where E, is the enzyme; Hx-R, is inosine; Pi, is inorganic phosphate; R-1-P, is ribose-1-phosphate; and Hx, is hypoxanthine.

The Michaelis constants for inosine were different for the vegetative cell and spore enzymes. The value for the spore enzyme was 1.5-fold greater than that found for the vegetative cell enzyme and both enzymes had greater Michaelis constants than that found for the erythrocyte enzyme (38). These differences may reflect subtle structural differences between the cell and spore proteins.

The concave downward curvature of the Lineweaver-Burk plots for both enzymes with inosine as the changing fixed substrate and phosphate as the variable substrate was unusual. This indicated an increase in maximal velocity and/or an increase in the Michaelis constant for phosphate at low levels of inosine. Plots of initial velocity vs. phosphate concentration were not sigmoidal but were rectangular hyperbolas. Therefore, the possibility of a classical allosteric effect was not supported. An allosteric enzyme has an effector binding

site, different from the active site, which non-competitively binds molecules other than substrate. This binding may decrease or increase enzyme activity. If the assumption is made that the maximal velocity for the vegetative cell and spore PNPases does not change (ie., the line on the replot of the intercepts vs. the reciprocal of the inosine concentration is linear) then the downward curvature would be the result of an increase in the Michaelis constant for phosphate. This would seem to support the theory that inosine must bind to the enzyme before phosphate. In this case, the binding of inosine may cause a conformational change on the PNPase enzyme which allows more efficient binding of the phosphate. At low inosine concentrations this conformational change may not occur on every PNPase molecule in the solution. This would result in a greater average Michaelis constant for phosphate over the entire enzyme population. High phosphate concentrations could also sterically hinder the binding of limiting amounts of inosine if the binding sites for the two substrates were proximally located. This hinderance would cause a decrease in the maximal velocity. The reverse effect was obtained, however.

Kim, et al. (38) attributed the non-linearity of the Lineweaver-Burk plot to a possible cooperative interaction between subunits. Two types of subunit

interaction have been reported which could explain these results. In the first type, an enzyme's catalytic activity varies with the number of subunits per active molecule due to changes in subunit association-dissociation equilibria (30). The activity of the individual subunits is assumed to be different from that found when the subunits are combined. If this were the case for the vegetative cell and spore PNPase enzymes, the plot of specific activity vs. protein concentration would have shown curvature and the initial velocity of the reaction would not have been proportional over a wide range of enzyme concentrations. In the second type of interaction, the binding of one substrate at one site would decrease the binding affinity of the remaining sites due to negative homotropic interactions between active centers (15). Therefore, each enzyme molecule would have more than one active center per subunit; or more subunits, each having one active center. The resulting rate of the catalytic reaction would be maintained at a moderate level in the presence of low phosphate concentrations because of the rapid combination of the first phosphate molecule. As the phosphate concentration was increased the rate of the reaction would increase rapidly. Engel and Dalziel (15) stated that, "the effect is as if the number of binding sites increases with the (phosphate) concentration, the

successive sites having larger Michaelis constants for the (phosphate) and perhaps greater maximum turnover rates." The results obtained during this study are consistent with this mechanism.

Both the spore and vegetative cell PNPase enzymes had similar Michaelis constants for phosphate. These values were 5-fold greater than that obtained for human erythrocyte PNPase (3.2×10^{-4} M) (38). It would be anticipated on the basis of the Michaelis constants that phosphate binding to the cell and spore PNPase proteins would be very weak. Yet, a profound effect of phosphate ion was noted in the heat resistance and molecular configuration of the enzymes. It would appear that phosphate ion binds at sites on the PNPase other than the catalytic site.

Preliminary sucrose density gradient studies indicated that the crystalline human hemoglobin used as the sedimentation marker in the previous study (16) apparently underwent a decrease in sedimentation velocity with increasing concentrations of phosphate ion. Field and O'Brien (18) reported that the sedimentation coefficient of hemoglobin decreased with a decrease in the hydrogen ion concentration. This drop in sedimentation velocity was due to a decrease in average molecular weight without accompanying configurational changes (90). Since there was some question of the usefullness of

hemoglobin as a marker in sucrose density gradient sedimentation studies, rabbit muscle lactic acid dehydrogenase was chosen. This enzyme could be easily assayed and had a reported sedimentation coefficient of 7.6 S (62).

Data obtained from the sucrose density gradient sedimentation studies of vegetative cell and spore PNPase enzymes, in the presence or absence of phosphate, did not coorelate with those reported previously (16). This discrepancy may be explained by changes in the sedimentation coefficient of the hemoglobin marker previously used. However, the general trend toward higher sedimentation velocities for the spore PNPase in phosphate was confirmed. The sedimentation velocity of the vegetative cell enzyme did not appear to change regardless of the phosphate ion concentration.

It was found that the increase in sedimentation velocity for the spore enzyme in the presence of phosphate ion may be irreversible. Spore PNPase which was dialyzed against buffer containing 10 mM potassium phosphate and then dialyzed against the standard buffer did not have a sedimentation coefficient corresponding to the value obtained in the standard buffer. The unique binding of phosphate which produced an enzyme with a greater sedimentation coefficient may be quite strong. This was in contrast to the phosphate binding which occurred in the catalytic reaction, with a Michaelis constant of about 1.3×10^{-3} M.

The striking result of the sucrose gradient studies was the comparatively small difference in sedimentation coefficients between the vegetative cell and spore enzymes in the standard buffer. Since it was known that the spore enzyme was significantly smaller than the vegetative cell enzyme, the similar sedimentation coefficients could only be explained by large conformational differences between the two enzymes. This conclusion was borne out by noting the constancy of the shape factor (f/f_o) for the vegetative cell-derived PNPase, in contrast to the increased value of the same parameter for the spore PNPase in 10 mM phosphate ion.

The high speed sedimentation equilibrium molecular weight study was made to obtain accurate molecular weight data. The plots of the natural logarithm of the fringe displacement vs. the squared distance from the center of rotation were nearly linear. Thus, the data were consistent with expected results and the molecular weight values can be accepted with certainity. If dissociation had occurred at the lowest concentrations, the plots would have shown significant curvature. The preparations also appeared to be nearly homogeneous since the experimental points along each plot did not show large deviations from the slope.

In the standard buffer (50 mM Tris-HCl, 10 mM 2-mercaptoethanol, pH 7.5), the spore PNPase had a lower

whole-cell average molecular weight than the vegetative cell enzyme. After the spore enzyme was dialyzed against buffer containing 10 mM potassium phosphate it had an average molecular weight which approximated that found for the vegetative cell PNPase. The nature of the phosphate-dependent molecular weight change for the spore enzyme can be found by examination of the weight-average (Mw) and number-average (Mn) molecular weight data. The spore PNPase preparation, in either the presence or absence of phosphate, contained more than one molecular weight component since the Mw values were greater than those for Mn. Only one molecular weight component was present in the vegetative cell preparation since the Mw and Mn values were nearly equal.

A previous sedimentation equilibrium study performed under similar conditions with a greater protein concentration of spore enzyme indicated that the preparation was not purely disperse. In a disperse system, the enzyme solution would have contained polymers that were not in chemical equilibrium with each other. In that case, the resulting plot of the ln of the fringe displacement vs. $(\text{radius})^2$ would have shown a curvature that differed from that found at the lower protein concentration (100). The slope, however, was similar and was shifted toward the top of the sector cell. This result would be expected for an associating system.

Since the spore PNPase preparations were not disperse and the protein concentrations at the meniscus were negligible (no changes in refractive index near the tops of the sector cells), an estimation of the molecular weight of the smallest species present in the solutions could be made. If sharp differences had been found between the slopes of ln D vs. (r^2) near the top and bottom of the sector cells the size of the smallest species could have been obtained by direct observation. Direct estimation is useful only if the solutes contain the smaller molecular weight component contaminated with polymers greater than trimer (100). Therefore, the plots obtained in this investigation suggested the presence of a polymer consisting of less than four of the smaller molecular weight components. The size of the spore PNPase enzyme in the absence of phosphate ion could be estimated from the relationship derived by Yphantis (100);

$$\text{Molecular weight} = \frac{j\,(Mn) - Mw}{j - 1}$$

where j, is the number of the smaller molecular weight components in the polymer; Mw, is the extrapolated weight-average molecular weight at zero protein concentration; and Mn, is the extrapolated number-average molecular weight at zero protein concentration. The

weight fraction of the polymer was assumed to be less than 25% near the top of the sector cell. By assigning various values for j ranging from 2 to 4, different minimal molecular weights were calculated: At j = 2, the molecular weight was 46,300; at j = 3, the minimal weight was 50,000; and at j = 4, the weight was 50,300. Since the whole-cell average molecular weight for spore PNPase in the absence of phosphate ion was 69,900 it was apparent that small amounts of polymer were present in the preparation. A measure of the spread of polymer distribution was obtained from the following relationship (90);

$$Mw/Mn = 1 + p$$

where p, is the extent of polymerization in a two component system. The value for p was 14%. Since the whole-cell average molecular weight for spore PNPase in the presence of 10 mM potassium phosphate was 94,800 it appeared that dimerization had occurred. When j was assigned a value of 2, the resulting calculated minimal molecular weight corresponded to a dimer having a molecular weight of 92,600. When higher values were assigned to j, the resulting molecular weights were greater than 100,000. The whole-cell molecular weight for the vegetative cell PNPase, in the absence and presence of phosphate, ranged between 88,300 and 91,600.

Thus, the spore PNPase appeared to undergo dimerization in the presence of phosphate ion to a size which approximated that of the vegetative cell enzyme.

The subunit analysis of the vegetative cell and spore enzymes indicated that both preparations had a subunit size of 24,000 ± 10%. Therefore, the process which converts vegetative cell PNPase to spore enzyme does not appear to be the result of gross proteolysis, but rather the rupture of a relatively few bonds in the vegetative cell protein. The subunit size agreed well with the calculated molecular weights of each enzyme. The subunit data indicated that spore PNPase in the absence of phosphate had two SDS-dissociable polypeptide chains whereas the vegetative cell PNPase had four.

The calculated f/f_o ratios indicated that spore PNPase in the standard buffer was more spherical (f/f_o = 1.2) than it was in the presence of phosphate ion. In 10 mM phosphate the ratio was the same as that found for the vegetative cell PNPase in the presence or absence of phosphate (f/f_o = 1.4). The axial ratios (78) of the spore monomer and dimer were about 4 and 8 respectively. This was consistent with the subunits joining end-to-end, if large conformational changes did not occur during the dimerization process. Laskowski (45) has stated that, ". . . a protein with about 500

amino acid residues could form one single glop of roughly spherical shape and still have enough surface to accommodate its charged residues; beyond this size spherical proteins appear impossible." The increase in the f/f_o ratio associated with dimerization of the spore PNPase is consistent with this view.

Proteins may be more heat stable when they are in a compact conformation since interactions between the protein and the surrounding environment would be reduced (90). Hydrophobic bonding may be the mechanism for this increased heat resistance. Hydrophobic bonds result from the negative affinity of non-polar protein side chains for water. This results in a tight packing of these non-polar groups into the interior of the protein molecule where they are stabilized by hydrophobic bonds. There is some question whether these bonds are a separate entity in themselves or actually the result of Van der Waals forces between closely associated side chains. Other bonds, such as hydrogen and ionic bonds, may contribute to protein stability but they do not seem to be a major factor in protein denaturation by agents such as urea.

The *in vivo* heat resistance of spore PNPase may be related to the phosphate effects which have been observed *in vitro*. Nelson, et al. (67) have shown that very little free phosphate is present in resting spores.

Therefore, the PNPase in spores may exist as nearly spherical monomers.

SUMMARY

The purine nucleoside phosphorylase from vegetative cells and spores of Bacillus cereus T was purified to electrophoretic homogeneity by ion exchange chromatography and polyacrylamide gel electrophoresis. The specific activity of these preparations was higher than those previously reported. Phosphate ion caused an increase in the thermal stability of both purified enzymes whereas inosine had no effect. The catalytic properties of the two enzymes were similar and indicated an ordered, sequential reaction mechanism where inosine was bound before phosphate. The Michaelis constants (inosine as substrate) for the vegetative cell and spore enzymes were 4.6×10^{-4} M and 7.0×10^{-4} M respectively. The Michaelis constant for phosphate was 1.5×10^{-3} M for the vegetative cell enzyme and 1.3×10^{-3} M for the spore enzyme. Both enzymes had similar turnover numbers and the specific activities of the enzymes appeared to be constant at varying protein concentrations. Negative homotropic effects were proposed since the Lineweaver-Burk plots with inosine as the changing fixed substrate and phosphate as the variable substrate showed downward curvature at high phosphate concentrations.

Sucrose density gradient studies indicated that spore purine nucleoside phosphorylase (PNPase) increased from a sedimentation velocity of 5.3 S in the absence of phosphate to a value of 5.7 S in the presence of 10 mM potassium phosphate. The increase in sedimentation velocity may have been irreversible. The vegetative cell PNPase had a sedimentation velocity of 5.5 S in the presence or absence of phosphate ion. The calculated minimal molecular weight of spore PNPase was 46,300 in the absence of phosphate, and 92,600 in the presence of 10 mM phosphate. The vegetative cell enzyme had a molecular weight of 88,300 and 91,600 in the presence and absence of phosphate respectively. The subunit size for both enzymes was 24,000 $\pm$ 10%. The conformational structure of the spore enzyme was more compact in the absence of phosphate ion. Dimerization of the spore enzyme to a molecular weight approximating the size of the vegetative cell enzyme was accompanied by a change in comformation.

Although the synthesis of both the vegetative cell and spore enzymes was directed by the same genomic unit, the enzymes differed in size and shape when isolated in the absence of phosphate ion.

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APPENDICES

APPENDIX A

MATERIALS USED IN THE INVESTIGATION

Acrylamide; N, N'-methylenebisacrylamide (bisacrylamide); glycine; 2-mercaptoethanol; and N, N, N'-tetramethylethylene diamine (TEMED) were purchased from Eastman Organic Chemicals. Inosine, hypoxanthine, xanthine, and dithiothreitol were obtained from Calbiochem. Rabbit muscle lactate dehydrogenase (LDH, specific activity of 150 units per mg), streptomycin sulfate, riboflavin, and trishydroxymethylaminomethane (Tris) were products of the Nutritional Biochemical Company. RNase and DNase I were purchased from Worthington Biochemical Corporation. Analytical reagent grade sucrose was prepared by Mallenckrodt, and Antifoam B emulsion by Dow Corning. Sodium lauryl sulfate (SDS) was a product of Sigma Chemicals. The #110 glass pavement marker beads were purchased from the Minnesota Mining and Manufacturing Company. Sephadex G-25 was a product of Pharmacia Fine Chemicals. The 2, 3, 5-triphenyltetrazolium chloride was from General Biochemicals, and Reeve Angel supplied the Whatman DE-52 microgranular DEAE-cellulose. The coomassie

brilliant blue was from Mann; the brom phenol blue was a product of Difco and the buffalo black was from Allied Chemical. All other materials used were of the highest grade obtainable.

APPENDIX B

DERIVATION OF THE FUNDAMENTAL SEDIMENTATION EQUATION

The sedimentation equilibrium method for molecular weight determination is based on the theory that at equilibrium the flow of solute due to sedimentation (Jsed) at every point in the cell is balanced by the counterflow due to diffusion (Jdiff).

Jsed = velocity + solute concentration

and, Centrifugal force = $m\omega^2 r'$

Taking into consideration the boyant force exerted by the solvent $(1 - \bar{v}\rho)$;

Centrifugal force = $m (1 - \bar{v}\rho) \omega^2 r'$

where m, is the effective mass of the solute particle; ω, is the angular velocity; r', is the distance from the center of rotation; $\bar{v}$, is the partial specific volume displaced by the solute; and ρ, is the density of the solvent. Since effective mass (m) is equal to the molecular weight of a single solute molecule (M) divided by Avogadro's number (N), the centrifugal force equation can be rewritten:

$$\text{Centrifugal force} = \frac{M\,(1 - \bar{v}\rho)\,(\omega^2 r')}{N} \qquad (I.)$$

and, Flow velocity = dr/dt = centrifugal force/f (II.)

where f, is the frictional coefficient of the solute particle. The frictional coefficient is defined as;

$$f = 6\P\eta r$$

where η, is the viscosity of the solvent; and r, is the effective radius of the protein.

Combining equations (I.) and (II.) gives the flow velocity:

$$\frac{dr}{dt} = \frac{M\,(1 - \bar{v}\,\rho)\,(\omega^2 r')}{N\,f}$$

Therefore;

Jsed = flow velocity x concentration of solute (c),

$$\text{or,} \qquad Jsed = \frac{M\,(1 - \bar{v}\,\rho)\,(\omega^2 r')}{N\,f} \cdot c \qquad (III.)$$

From Fick's first law of diffusion;

$$Jdiff = - D\, dc/dr \qquad (IV.)$$

where D, is the diffusion coefficient of the solute; and dc/dr, is the solute concentration gradient; and from the Einstein diffusion equation;

$$D^{\circ} = \frac{R\ T}{N\ f} \qquad \text{(V.)}$$

where R, is the universal gas constant; and T, is the absolute temperature, the force due to diffusion is derived. Combining equations (IV.) and (V.) gives:

$$Jdiff = - \left(\frac{R\ T}{N\ f}\right) \cdot \frac{dc}{dr} \qquad \text{(VI.)}$$

At sedimentation equilibrium, Jsed = Jdiff. Therefore, combining equations (III.) and (VI.) gives;

$$\frac{dc}{dr} = \frac{M\ (1 - \bar{v}\ \rho)\ \omega^2 r'\ c}{R\ T} \qquad \text{(VII.)}$$

the fundamental sedimentation equilibrium equation for a single solute in an ideal solution.

Determination of Molecular Weights From Actual Data

From the integration of the fundamental equation (VII.), it follows that:

$$\ln c = \frac{M\ (1 - \bar{v}\ \rho)\ \omega^2 r'^2}{2\ R\ T} + c \qquad \text{(VIII.)}$$

Given that:

$$n = n_o \, k \, c \qquad \text{(IX.)}$$

where n, is the refractive index of the solute; n_o, is the refractive index of the solvent; k, is a constant; and c, is the solute concentration, the interference fringes from each sector cell give an indication of the protein concentration at each point in the cell. By combining equations (VIII.) and (IX.) the relationship between protein concentration and molecular weight at equilibrium is established:

$$\ln (n - n_o) = \frac{M (1 - \bar{v} \rho) \omega^2 r'^2}{2 R T} + c$$

If a plot is made of $\ln (n - n_o)$ vs. r'^2, the slope is equal to the following;

$$\frac{M (1 - \bar{v} \rho) \omega^2}{2 R T}$$

which allows a solution for average molecular weight. The computer program was designed to take five equally spaced adjacent data points along the X-coordinate and determine the least square straight line through these points. The slope of this line was equal to the slope at the central point given by fitting a least square quadratic through the same five points. The unweighted least squares

procedure, using five points at a time, produced an average least square straight line through the average slopes obtained for each set of five points over the entire cell. The molecular weights obtained by this procedure were very accurate.

The computer was also programmed to analyze the data and compute weight-average (Mw) and number-average (Mn) molecular weights at every data point in the cell. From these calculated values plots were constructed of either Mw or Mn vs. protein concentration. When the points on each plot were connected by a least square line and extrapolated to zero protein concentration, the values for Mw and Mn of the smallest protein moiety were obtained. The mathematical operations used in these calculations are reviewed by Yphantis (100).

APPENDIX C

CALCULATION OF FRICTIONAL COEFFICIENTS

The f/f_o ratios derived from the molecular weight and sedimentation coefficient data were calculated in the following manner using the fundamental relationship between molecular weight and sedimentation velocity;

$$S^o = \frac{M\ (1 - \bar{v}\ \rho)}{N\ f} \qquad \text{(I.)}$$

From Stoke's law;

$$f = 6\ \P\eta r$$

which is defined in Appendix B. From the volume of a sphere (V);

$$V = 4/3\ \P r^3$$

where,

$$r = \left(\frac{3\ V}{4\ \P}\right)^{1/3} \qquad \text{(II.)}$$

and from the volume of a spherical protein (V^1);

$$V' = \frac{M\,\bar{v}}{N} \qquad \text{(III.)}$$

the radius of a spherical protein (r), can be derived by combining equations (II.) and (III.):

$$r = \left(\frac{3\,M\,\bar{v}}{4\,N\,\P}\right)^{1/3} \qquad \text{(IV.)}$$

Combining equations (I.) and IV.), gives the fundamental relationship between molecular weight and sedimentation coefficient for a spherical protein under ideal conditions:

$$S^{\circ} = \frac{M\,(1 - \bar{v}\,\rho)}{6\,N\,\P\,\eta\left(\frac{3\,M\bar{v}}{4\,N\P}\right)^{1/3}} \qquad \text{(V.)}$$

This equation was solved, assigning a value of 0.717 ml/gm for the partial specific volume ($\bar{v}$) of PNPase. The values for the solvent density (ρ) and for the viscosity of the solvent (η) were known. The solution was;

$$S^{\circ} = 3.79 \times 10^{-3}\ M^{2/3}$$

Using this equation and the whole-cell average molecular weight values obtained from the sedimentation equilibrium study, sedimentation coefficients were calculated for both the vegetative cell and spore PNPase enzymes in the presence or absence of 10 mM potassium phosphate. These calculated sedimentation coefficients were the values which would be expected if the PNPase enzymes were spherical molecules.

Using a rearranged form of equation (I.);

$$f = \frac{M}{S} \cdot \left[\frac{(1 - \bar{v}\,\rho)}{N}\right]$$

the minimal frictional coefficients (f_o) were calculated from the whole-cell average molecular weight data and the calculated S^o values for each enzyme. Frictional coefficients were also calculated from the molecular weight data and the experimentally determined sedimentation coefficients to give the actual frictional coefficients (f) for each enzyme preparation. From the f/f_o ratios, an indication of the molecular asymmetry could be found. A spherical molecule would have a f/f_o ratio of one. Most proteins have ratios in the range from 1.0 to 2.0.