Purification and Properties of Vitamin B_6 Kinase from *Escherichia coli* B*

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ABSTRACT: Pyridoxine kinase from *Escherichia coli* B was purified 3000-fold to apparent homogeneity. The enzyme had a molecular weight of 35,000 by sedimentation equilibrium and 44,000 by sucrose density gradient centrifugation. Kinetic studies with new spectrophotometric assays showed that the reaction required ATP, one of the pyridoxine substrates, and either Zn^{2+} or Mg^{2+}. The optimum concentrations for Zn^{2+} and Mg^{2+} were 5×10^{-4} and 1×10^{-3} M, respectively. The velocity of the reaction at the optimum Zn^{2+} concentration was twice the velocity of the reaction at the optimum Mg^{2+} concentration.

The $K_{m,app}$ for pyridoxal, as determined by direct spectrophotometry at 388 nm, was 3×10^{-4} M, and the $K_{I,app}$ values of pyridoxol, pyridoxamine, and 5-deoxypyridoxal as competitive inhibitors were 8×10^{-6}, 5×10^{-4}, and 2×10^{-4} M respectively. The K_i values of pyridoxal 5′-phosphate, pyridoxol 5′-phosphate, and pyridoxamine 5′-phosphate (as noncompetitive inhibitors) were 4×10^{-4}, 2×10^{-3}, and 4×10^{-3} M, respectively. The kinase was strongly inhibited by pyridoxal.

Pyridoxine[1] kinase was first extensively studied by Hurwitz (1953) who partially purified the enzyme from brewers' yeast. He determined not only that the stoichiometry of the kinase reaction required a 1:1 molar ratio of pyridoxal to ATP, but also that the partially purified enzyme required a divalent cation for activity. In addition he showed that the kinase phosphorylated both pyridoxol[2] and pyridoxamine, as well as pyridoxal.

McCormick *et al.* (1961) using partially purified kinases from several sources showed that these kinases phosphorylated each of the pyridoxine substrates, although the concentrations of substrates required for optimal phosphorylation depended on both the source of the enzyme and the substrate under study. For example, the kinase from yeast was found to phosphorylate pyridoxol and pyridoxamine at significantly lower concentrations than pyridoxal. In contrast, the kinases from two bacterial sources phosphorylated pyridoxal at much lower concentrations than they did pyridoxol or pyridoxamine. In addition, the maximum velocities of phosphorylation obtained with each of the pyridoxine substrates at their optimal concentrations were approximately the same.

Recently Tsubosaka and Makino (1969) reported a 3500-fold purification of this enzyme from mouse brain. Studies made with this 60–70% pure kinase yielded $K_{m,app}$ values of 8.6×10^{-5}, 3×10^{-4}, 5×10^{-5}, and 2×10^{-4} M for pyridoxal, toxopyrimidine, pyridoxol, and pyridoxamine, respectively.

We report here the purification to constant specific activity of the pyridoxine kinase from *Escherichia coli* B, the development of spectrophotometric assay procedures which allow measurement of initial velocities of the kinase reaction without the limitations of the previous stepwise assay, and the discovery that pyridoxine kinase is inhibited by pyridoxal.

Materials and Methods

Bacteria. E. coli B, "three-fourth grown in enriched medium" was purchased as a frozen paste from Grain Processing Corp., Muscatine, Iowa.

Chemicals. Pyridoxol-HCl, pyridoxal-HCl, pyridoxamine-HCl, adenosine 5′-triphosphate (Na_2 salt), and reduced glutathione were purchased from Sigma Chemical Co. Pyridoxal 5′-phosphate monohydrate was purchased from Mann Research Laboratory, Inc. Pyridoxamine 5′-phosphate and pyridoxol 5′-phosphate were purchased from Calbiochem. Phosphate esters of the vitamin B_6 family were purified by the ion-exchange chromatographic method of Bain and Williams (1960) before use. Pyridoxamine was purified as described below. DEAE-cellulose type 40 was used for enzyme purification after repeated cycling through NaOH and HCl according to the method of Peterson and Sober (1962). Enzyme grade sucrose and ammonium sulfate were purchased from Mann Research Laboratories. Sephadex G-100 was purchased from Pharmacia Inc. and divided into five particle sizes by sieving. Only particles between 88 and 105 μ were used.

Enzymes. Catalase and horseradish peroxidase were purchased from Worthington Biochemicals. Malic dehydrogenase, lactic dehydrogenase, and pyruvate kinase were all obtained from C. F. Boehringer und Soehne. Rabbit liver pyridoxine phosphate oxidase was purified through the Alumina Cγ step of Wada and Snell (1961) and stored at −20°.

Cation-Exchange Chromatography of Pyridoxamine Di-

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[1] Pyridoxine is used here to refer to the three nonesterified forms of vitamin B_6.

[2] Pyridoxol refers specifically to the 2-methyl-3-hydroxy-4,5-dihydroxymethylpyridine vitamer.

hydrochloride. Commercial pyridoxamine dihydrochloride was purified using the following method of J. B. Lyon (personal communication). A 1 × 12 cm column of Bio-Rad AG 50-X8 (K^+, 200–400 mesh) was washed with 0.02 M potassium formate (pH 4.2) until the pH of the eluate measured 4.2. A 100-mg sample of pyridoxamine dihydrochloride dissolved in 10 ml of the formate buffer was applied to the column and the column was washed with 50 ml of the same buffer. A gradient was applied by passing 0.02 M citrate (pH 6.5) 0.2 M in KCl through a 300-ml constant-volume mixing chamber containing 0.02 M potassium formate (pH 4.2). The eluate from 260 to 330 ml was pooled, diluted 1:3 with water, and applied to a second column equilibrated with 0.02 M potassium formate (pH 4.2). The second column was washed extensively with deionized water and the pyridoxamine subsequently eluted in a small volume with 0.3 M NH_4OH. The sample was dried under reduced pressure, then repeatedly redissolved in H_2O, and dried to remove the ammonia. The pyridoxamine was dissolved in ethanol, filtered, and dried under reduced pressure over P_2O_5.

Sucrose Gradient Centrifugation. Liner sucrose gradients from 5 to 20% sucrose in 0.02 M potassium phosphate buffer at pH 7.5 were prepared to a total volume of 4.8 ml using a gradient mixing device similar to the one described by Martin and Ames (1961). The gradients were stored at 4° for 4–6 hr prior to use. A sample (0.01 ml) which contained the purified kinase and molecular weight standards (less than 2% total protein) was dialyzed against 0.02 M potassium phosphate buffer (pH 7.5) and then carefully layered on each gradient. The gradient-containing tubes were centrifuged for 18 hr at 37,500 rpm in the SW-39 rotor of a Spinco L2-65 preparative ultracentrifuge (5°). Malic dehydrogenase (mol wt 70,000) and peroxidase (mol wt 40,000) were used as standard molecular weight markers. Approximately 26 fractions of 0.2 ml each were collected and assayed for activity as described below.

Malic dehydrogenase activity was measured by the method of Ochoa (1959). Horseradish peroxidase activity was measured by following the changes in extinction at 420 nm in a 1-cm cuvet containing 0.4 ml of 0.04 M H_2O_2, 0.3 ml of 0.05 M $K\text{-}PO_4$ (pH 7.2), 0.1 mg of *O*-dianisidine, and H_2O plus enzyme to a total volume of 1.5 ml.

Optimal Assay Procedures for Pyridoxine Kinase from E. coli. For the kinase–apotryptophanase assay, the following amounts of reagents were incubated in small stoppered test tubes at 37° for 15 min: 0.1 μmole of ATP, 0.1 μmole of pyridoxal-HCl, 1.0 μmole of $ZnSO_4$, 10 μmoles of potassium phosphate (pH 7.0), varying concentrations of enzyme, and water to a final volume of 1 ml. The reactions were started by the addition of enzyme to each tube and terminated after 15 min by heating in a boiling-water bath for 5 min. Controls measuring endogenous pyridoxal 5′-phosphate lacked only pyridoxal. Aliquots from each kinase reaction tube and a series of pyridoxal 5′-phosphate standards from 0 to 6 nmoles were added to 25-ml flasks, and assayed by the apotryptophanase assay as described by McCormick *et al.* (1961).

For the assay using direct spectrophotometry at 388 nm, an aliquot containing 0.001–0.010 unit of a 200-fold or greater purified pyridoxine kinase preparation was added to a cuvet containing 1 μmole of ATP, 1 μmole of pyridoxal-HCl, 10 μmoles of potassium phosphate (pH 6.0), 0.5 μmole of $ZnSO_4$, and water to a final volume of 1.0 ml. The increase in absorbance at 388 nm was followed with time in a recording spectrophotometer with the scale adjusted from 0 to 0.1 optical density unit for full-scale deflection.

For the coupled pyridoxine kinase–ADP assay, an aliquot containing 0.001–0.01 unit of a 200-fold- or greater than 200-fold-purified pyridoxine kinase preparation was added to a cuvet containing 15 μmoles of potassium phosphate (pH 6.0), 25 μmoles of KCl, 1 μmole of tricyclohexylammonium phosphoenolpyruvate, 5 μmoles of $MgCl_2$, 1.0 μmole of ATP, 0.10 μmole of pyridoxine substrate, 0.1 μmole of NADH, excess pyruvate kinase, lactic dehydrogenase, and water to make a final volume of 1.0 ml. The decreases in absorbance at 340 nm due to NADH oxidation were followed with time in a double-beam spectrophotometer (full-scale absorption from 0 to 0.1 optical density), and the difference between the rate prior to and that subsequent to enzyme addition was considered to be equal to the net pyridoxine kinase rate.

Results

Assays. The assay commonly used for pyridoxal kinase is a stepwise one in which mixtures containing kinase, ATP, pyridoxal, and a metal ion are incubated to allow formation of pyridoxal 5′-phosphate. The kinase activity is then destroyed by heating and the amount of pyridoxal 5′-phosphate formed is measured by allowing a portion of the heated mixture to convert some apotryptophanase into holotryptophanase. The extent of this conversion, measured by the amount of indole formed in 15 min from tryptophan by the catalytic action of holotryptophanase, is then related to the amount of pyridoxal 5′-phosphate originally present. To test either pyridoxol or pyridoxamine as kinase substrates requires another step between the kinase and the apotryptophanase reactions. In this step rabbit liver pyridoxol 5′-phosphate oxidase is used to convert any pyridoxol 5′-phosphate and pyridoxamine 5′-phosphate formed by the kinase to pyridoxal 5′-phosphate (Wada and Snell, 1961).

For kinetic studies, this assay procedure has a major drawback which stems from the necessity of stopping the kinase reaction by heat denaturation. For *E. coli* B pyridoxine kinase, stopping the reaction by heating in a boiling water bath takes from 2 to 5 min depending on the purity of the enzyme.

In order to reduce the effect this heating time has on the determination of a precise rate it would be necessary to use very dilute enzyme and long incubation times. While the instability of dilute pyridoxal 5′-phosphate solutions alone would make this approach unsatisfactory, a much more serious objection to prolonged incubation of kinase reaction mixtures arises from the finding reported below that pyridoxal slowly but very effectively inhibits the kinase. Dilute kinase solutions therefore would be very badly inhibited by substrate during long incubations.

The above problems can be avoided if the kinase reaction is assayed directly by following increases in absorbancy at 388 nm since pyridoxal 5′-phosphate absorbs strongly at this wavelength at neutral pH, but pyridoxal does not. Although this assay is not as sensitive as the above assay, it is linear in response to kinase and allows a simple, direct measurement of initial rate of reaction. It should be pointed out however that this assay cannot be used with pyridoxol or pyridoxamine because these substrates do not form products which have significantly different absorption spectra from their substrates.

An assay which also allows direct spectrophotometric measurement of the initial rate of pyridoxine kinase without restricting the nature of the pyridoxine substrate is one in which ADP formation is coupled to DPNH oxidation in a mixture containing pyruvate kinase and lactic dehydrogenase, as described by Uyeda and Racker (1965). Decreases in absorbancy at 340 nm then reflect kinase activity. This assay is also linear in its response to changes in sample size.

Although serious objections can be raised against the use of the sequential kinase–apotryptophanase assay in kinetic studies, this assay remains the most sensitive of the three assays. Accordingly, we adopted it for routine use during purification of pyridoxine kinase and reserved the spectrophotometric assays for kinetic studies. The optimal conditions for each assay are described in the Methods section. For both the sequential kinase–apotryptophanase assay and the assay measuring pyridoxal 5′-phosphate directly at 388 nm, these conditions were determined with pure enzyme. Conditions for the ADP-coupled assay were determined only with partially purified kinase.

Definition of Unit of Specific Activity. With all three pyridoxine kinase assays the specific activities of the kinase are expressed as units per mg of protein. In all cases protein concentration was determined by the method of Lowry *et al.* (1951) using crystalline bovine plasma albumin as the standard. For the kinase–apotryptophanase assay, one unit is the amount of enzyme that forms 1 μmole of pyridoxal 5′-phosphate/min at 37°. The amount of pyridoxal 5′-phosphate formed per minute was determined from the total micromoles formed in a 15-min incubation time, even though the increase in pyridoxal 5′-phosphate is not linear over the entire time interval. For direct spectrophotometry at 388 nm, one unit is defined as the amount of enzyme that forms 1 μmole of pyridoxal 5′-phosphate/min at room temperature. The micromoles of pyridoxal 5′-phosphate formed were determined from the experimentally determined molar extinction coefficient at 388 nm of 4900 for pyridoxal 5′-phosphate at pH 6.0. For the ADP assay, one unit is the amount of enzyme that forms 1 μmole of NAD/min at room temperature (25°). The micromoles of NAD formed were determined from the molar extinction coefficient of 6200 for NADH at 340 nm (Uyeda and Racker, 1965).

Purification. Approximately 260 g (dry weight) of *E. coli* B, suspended in approximately 1.5 l. of 0.01 M potassium phosphate (pH 7.0), were disrupted at 5° by ultrasonic oscillation at 20,000 Hz until the maximum amount of protein was released. The average time for this was 2–3 hr. Foam was retarded with General Electric Antifoam 60. The mixture was centrifuged at 25,000*g* for 3 hr and the resulting precipitate was discarded. Room temperature saturated ammonium sulfate (at pH 7.2) was added dropwise to the supernatant solution at 2° until 30% saturation (calculated for 25°) was reached. The resultant solution was stirred overnight at 2–4° and centrifuged 45 min at 25,000*g*. The precipitate was discarded and the supernatant fluid was brought to 52% saturation by further addition of ammonium sulfate and stirred 2 hr at 2–4°. The precipitate obtained after centrifugation at 25,000*g* for 45 min was dissolved in 0.01 M potassium phosphate (pH 7.0) and stored at 2°. Four additional 260-g batches of *E. coli* were then brought to this stage and pooled.

Stepwise DEAE-cellulose Column Chromatography. The pooled 30–52% ammonium sulfate fractions were dialyzed exhaustively against 0.01 M potassium phosphate buffer (pH 7.0) until the ammonium ion concentration in the dialysate after 8-hr equilibration was less than 0.01 M as determined by Nessler's reaction. The dialyzed material was applied to an 8 × 100 cm DEAE-cellulose column equilibrated with 0.01 M potassium phosphate buffer (pH 7.0). The column was then washed with approximately 2 l. of that buffer until the first protein peak, as measured by absorbancy at 280 nm, was eluted.

After this, 0.01 M potassium phosphate (pH 7.0), 0.08 M in KCl, was applied until all the kinase peak washed off. Fractions (150) of 20 ml were collected during this time and the 50 fractions containing the most units of kinase were concentrated to 80 ml in an ultrafiltration apparatus containing a Diaflo UM-10 membrane with an apparent pore radius of 10 Å. The concentrated protein was applied in two equal parts to two separate 5 × 100 cm Sephadex G-100 columns equilibrated with 0.01 M potassium phosphate (pH 7.0). The protein was eluted by reverse flow of the buffer, and 11.0-ml fractions were collected from each. The seven most active fractions from both columns were combined.

DEAE-cellulose Column Chromatography. The Sephadex fractions were applied to a 0.9 × 80 cm DEAE-cellulose column equilibrated with 0.01 M potassium phosphate buffer (pH 8.0). The column was washed with 200 ml of that buffer and a 1-l. linear gradient of KCl from 0 to 0.5 M was applied. Fractions of specific activity 0.043 μmole/min per mg or greater were pooled, dialyzed overnight against 2 l. of 0.01 M potassium phosphate buffer (pH 6.0), and then applied to a 0.9 × 80 cm DEAE-cellulose column equilibrated with 0.01 M potassium phosphate buffer (pH 6.0). After the column was washed with 200 ml of that buffer, a 1-l. linear gradient was applied from 0 to 0.3 M KCl. Fractions (6.0 ml/tube) of specific activity 0.13 or more were pooled, dialyzed overnight against 2 l. of 0.01 M potassium phosphate buffer (pH 8.0), and applied to a 0.9 × 45 cm DEAE-cellulose column at pH 8.0. The column was washed with 200 ml of that buffer and a 800-ml linear gradient was applied from 0 to 0.4 M KCl. Fractions of 5 ml were taken. This purification scheme, summarized in Table I, resulted in an approximately 3000-fold purification of the kinase from sonicated cells.

Criteria for Homogeneity. The fractions of highest specific activity from the final DEAE-cellulose column exhibited a constant specific activity over four fractions (Figure 1). Polyacrylamide gel electrophoresis by the method of Davis (1964) of the fractions of highest specific activity showed a single band after staining (Figure 2).

Molecular Weight Determinations. The molecular weight of the purified pyridoxine kinase enzyme was estimated by the sucrose density gradient centrifugation method of Martin and Ames (1961) and by the sedimentation equilibrium method of Yphantis (1964). In the former method, the kinase molecular weight was calculated to be 44,000 ± 1000 by comparison of its sedimentation behavior in 20-hr sucrose density gradient centrifugations with the behavior of malic dehydrogenase (mol wt 70,000) and peroxidase (mol wt 40,000). In the latter method the kinase molecular weight was determined to be 35,000 from sedimentation equilibrium patterns obtained at a speed of 37,020 rpm in the Beckman Model E ultracentrifuge. The duration of each experiment was approximately 18 hr and the attainment of equilibrium was checked by comparison of traces taken at intervals of several hours. For each experiment the plot of the logarithm of the

TABLE I: Purification of Pyridoxine Kinase.[a]

	Total Units	Total mg of Protein	Vol (ml)	Sp Act. × 10^3	% Yield
Sonicated cells	80	840,000	8000	0.095	100
Centrifuged cells	73	510,000	7800	0.15	92
30–52% $(NH_4^+)_2SO_4$	57	190,000	2000	0.30	71
DEAE-cellulose, pH 7.0, stepwise	33	34,000	1000	0.97	41
DEAE-cellulose, pH 7.0, gradient	23	8,400	160	2.7	29
DEAE-cellulose, pH 6.0, gradient	19	4,200	130	4.4	23
Sephadex G-100 column	10	340	150	29	13
DEAE-cellulose, pH 8.0, 0.9 × 80 cm	4.9	60	30	75	6.1
DEAE-cellulose, pH 6.0, 0.9 × 80 cm	3.0	16	35	200	3.8
DEAE-cellulose, pH 8.0, 0.9 × 45 cm	2.3	8.5	35	270	2.9

[a] Units of kinase activity are expressed as micromoles of pyridoxal 5′-phosphate formed per minute in a 15-min incubation of the enzyme with 0.1 mM ATP, 0.1 mM pyridoxal-HCl, 1 mM $ZnSO_4$, and 0.01 M potassium phosphate buffer (pH 7.0). After the reaction was terminated with a 5-min boiling-water bath treatment, the pyridoxal 5′-phosphate was determined using the apotryptophanase reaction. *NOTE:* The specific activity of the final DEAE fractions (specific activity 0.270 by apotryptophanase) was 8 μmoles of pyridoxal 5′-phosphate/min per mg as determined by direct spectrophotometry described in Methods section.

fringe displacement against the distance from the axis of rotation yielded a straight line. A partial specific volume of 0.74 was assumed in order to calculate the kinase molecular weight. For the determination of an approximate kinase molecular weight, the values obtained by the two procedures were averaged to yield an approximate kinase molecular weight of 40,000.

Specific Activity Ratios of Pyridoxine Substrates. In order to test the hypothesis that a single kinase enzyme was responsible for the phosphorylation of all three substrates, we measured the specific activity of two kinase preparations with each of the three pyridoxine substrates. A comparison of the ratios of these specific activities showed that they did not change significantly between a preparation 100-fold purified and one 2500-fold purified, thereby suggesting that only one kinase capable of phosphorylating vitamin B_6 compounds at these rates was present.

Requirements for Phosphorylation. Kinetic studies with the newly developed spectrophotometric assays for pyridoxine kinase confirmed the findings made by others (Hurwitz, 1953; McCormick, *et al.*, 1961) with partially purified kinases from other sources, namely the reaction requires ATP, one of the nonphosphorylated vitamin B_6 compounds, and a divalent cation. Figure 3 indicates that the optimum concentrations for Zn^{2+} and Mg^{2+} are 5×10^{-4} and 1×10^{-3} M, respectively, in this direct assay. The maximum velocity at the

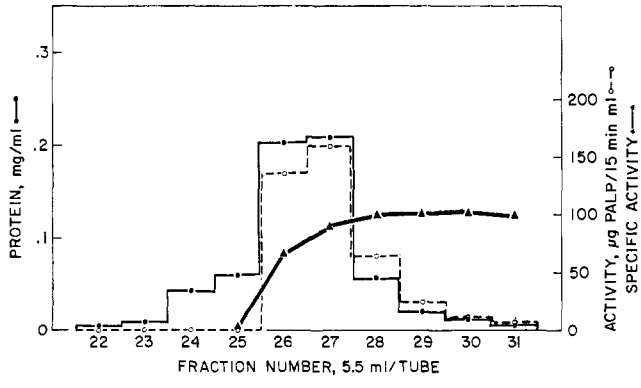

FIGURE 1: DEAE-cellulose column chromatography, pH 8.0, 0.9 × 45 cm column. Protein determinations and kinase assays were as described in Methods.

TABLE II: $K_{m,app}$ and K_i Values for Pyridoxine Kinase.

Compd Tested	Direct Spectrophotometry at 388 mμ		ADP Assay
	$K_{m,app}$ (M)	$K_{i,app}$ (M)	$K_{m,app}$ (M)
Pyridoxol		8×10^{-6}	7×10^{-6}
Pyridoxal	3×10^{-4}		
			1×10^{-4}
Pyridoxamine		5×10^{-4}	6×10^{-4}
5-Deoxypyridoxal		2×10^{-4}	
Pyridoxol 5′-phoshpate		2×10^{-3}	
Pyridoxamine 5′-phosphate		4×10^{-3}	
Pyridoxal 5′-phosphate		4×10^{-4}	

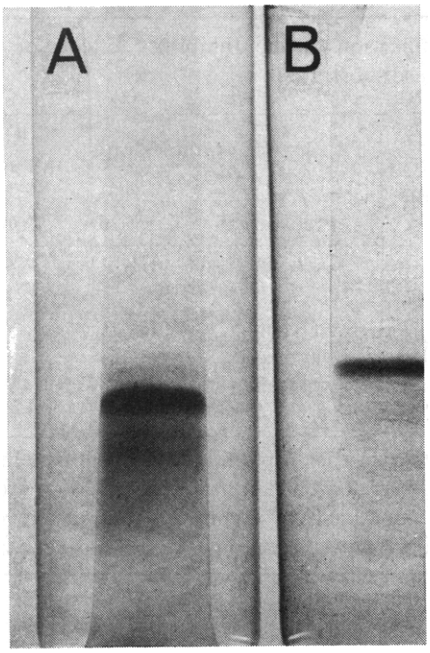

FIGURE 2: Polyacrylamide gel electrophoresis of the purified pyridoxine kinase enzyme. Acrylamide concentration was 7.5% and the pH of the Tris-glycine buffer was 8.5. In both A and B the protein concentration as measured by the method of Lowry was approximately 150 μg. (A) Stained gel of the fractions which contained a minor protein contaminant. (B) Stained gel of the fractions of highest (constant) specific activity (fractions 28, 29, 30, and 31 from the final DEAE-cellulose column). Differences in staining intensity as depicted above are because B is a picture of a gel only half of which was stained.

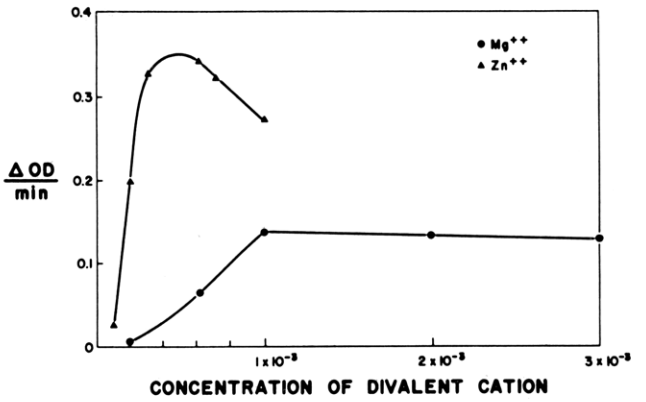

FIGURE 3: Effect of divalent cation concentration (Mg^{2+} and Zn^{2+}) on the velocity of phosphorylation. The increases in extinction at 388 mμ were followed in a Gilford recording spectrophotometer after the addition of 4 μg of purified kinase to a cuvet containing 0.2 mM pyridoxal, 0.2 mM ATP, 10 mM potassium phosphate buffer (pH 6.0) varying concentration of Zn or Mg divalent cation, and H_2O to a volume of 1 ml.

optimum Zn^{2+} concentration is twice the maximum velocity at the optimum Mg^{2+} concentration, and the presence of Mg^{2+} reduces the maximum velocity of the reaction seen with optimal Zn^{2+} concentration.

Apparent K_m and K_i Determinations. The initial velocities of phosphorylation at several concentrations of pyridoxal were determined by direct spectrophotometry at 388 nm and plotted in double-reciprocal plots against the substrate concentration (Figure 4). $K_{m,app}$ values determined from a number of such plots yielded an average $K_{m,app}$ for pyridoxal of 3×10^{-4} M. Similar plots of data obtained with constant concentrations of inhibitors showed that pyridoxol, 5-deoxypridoxal, and pyridoxamine were competitive inhibitors with the K_i values shown in Table II. In all experiments, the ATP concentration of 1×10^{-3} M was saturating and the concentration of Zn^{2+} was the optimum as determined from data plotted in Figure 3. The $K_{m,app}$ of the vitamers proved to be of the same order of magnitude as the K_i values when the coupled assay for ADP formation was used (Table II).

$K_{i,app}$ values (Table II) for the three products of the pyridoxine kinase reactions were determined from inhibition studies of pyridoxal phosphorylation. Double-reciprocal plots of velocity against substrate concentration, as shown in Figure 5, indicated that the presence of the products decreased the $V_{max,app}$ of pyridoxal but did not alter its $K_{m,app}$. Thus the products appeared to be noncompetitive inhibitors of pyridoxal phosphorylation.

Turnover Number. By direct spectrophotometry at 388 nm with pyridoxal as substrate the approximate turnover number for pyridoxine kinase was determined to be 350 moles of pyridoxal/min per mole of kinase at 37°. For these measurements, the optimal ATP and Zn^{2+} concentrations used were those determined at 25°. In a living *E. coli* cell, maximum turnover number might easily be as high as 525 moles of pyridoxol/min per mole of kinase at 37° since comparative rate studies with the coupled assay for ADP indicate that the maximum rate of phosphorylation of pyridoxol is 1.5 times that of pyridoxol. The difficulty in obtaining the maximum turnover number with pyridoxol arises from our inability to directly measure pyridoxol kinase activity by an assay procedure which contains the optimum Zn^{2+} concentration and no Mg^{2+}. Since pyruvate kinase requires Mg^{2+}, the maximum rate of pyridoxol phosphorylation might not be obtained in the coupled assay because of competition between the Zn^{2+} and Mg^{2+}.

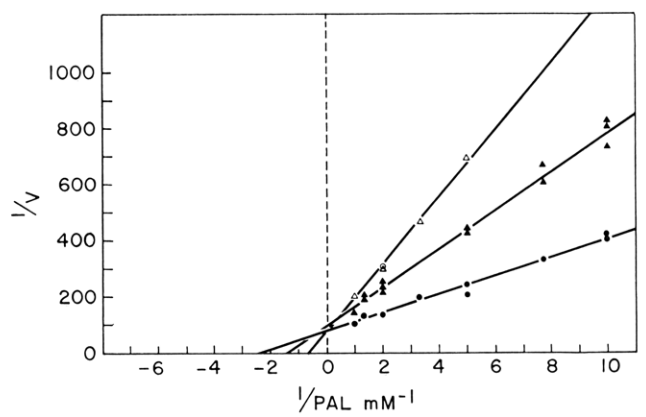

FIGURE 4: Double-reciprocal plots of initial velocities of phosphorylation with pyridoxal as the varied substrate. The increases in extinction were followed at 388 mμ in a 1-cm cuvet containing 1 mM ATP, 0.5 mM $ZnSO_4$, and 10 mM potassium phosphate (pH 6.0), varying concentrations of pyridoxal with a constant concentration of inhibitor (●, no inhibitor; ▲, 2×10^{-5} M pyridoxol; △, 3×10^{-4} M 5-deoxypyridoxal), and H_2O to a final volume of 1 ml. $1/V$ is expressed as the reciprocal of the optical density change per minute.

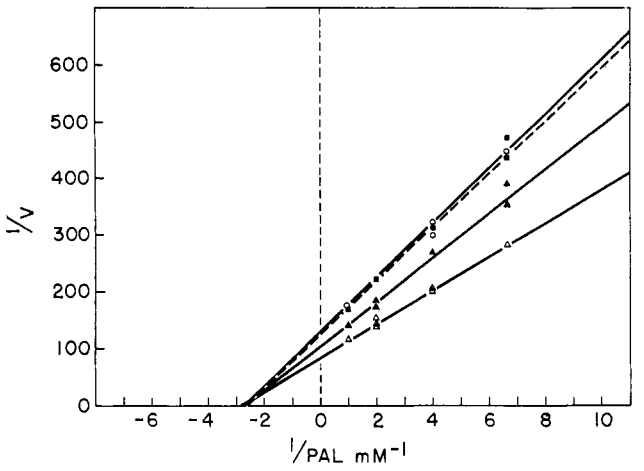

FIGURE 5: Effects of phosphorylated products on the apparent V_{max} of pyridoxal phosphorylation. Initial velocities of phosphorylation were measured as described in Figure 6: △, no inhibitor; ▲, 1×10^{-3} M pyridoxamine 5′-phosphate, ■, 1.8×10^{-4} M pyridoxal 5′-phosphate, and ○, 1×10^{-3} M pyridoxol 5′-phosphate. $1/V$ is expressed as the reciprocal of the optical density change per minute.

Pyridoxal Inhibition. Whenever we used the direct assay at 388 nm for pyridoxal 5′-phosphate formation we found that there was a significant effect of the order of addition of substrates upon the initial rate. The compound responsible for affecting the rate was pyridoxal and not ATP, because the specific activity of the enzyme decreased only with increasing time of exposure to pyridoxal. (This effect was not found with pyridoxol, pyridoxamine, or any of the three phosphate esters of vitamin B_6). Table III shows the inhibitory effect seen when the enzyme was exposed to 4×10^{-4} and 5×10^{-5} M pyridoxal for varying times. In these experiments, catalytic amounts of kinase in phosphate buffer (pH 6.0) containing 5×10^{-4} M $ZnSO_4$ were exposed to pyridoxal for the indicated times, then additional pyridoxal was added to bring the final concentration to 10^{-3} M and immediately thereafter ATP was added to a final concentration of 10^{-3} M to initiate the kinase reaction. Assay was made by following pyridoxal 5′-phosphate formation at 388 nm. Table III shows that the rate of pyridoxal 5′-phosphate synthesis by a kinase preparation exposed 30 min to 5×10^{-5} M pyridoxal was approximately 50% of the rate measured without preincubation with pyridoxal. The same effect to the same extent was observed with pyridoxal from two different commercial sources as well as with pyridoxal which was first recrystallized, converted into the free base, and then recrystallized again, thereby reducing the probability that the effect seen arose from a contaminant. Table IV shows that the inhibitory effect was not seen with either pyridoxol or pyridoxamine. In these cases kinase preparations were allowed to react with 5×10^{-5} M pyridoxol or pyridoxamine under conditions identical with those described above for pyridoxal, then pyridoxal and ATP were added to 0.4 mM as above to assay the activity. With both pyridoxol and pyridoxamine the kinase showed initial rates at least 90% of the control. The phosphate esters of vitamin B_6 were without effect in this test system. The reduced rate shown in Table IV for pyridoxol undoubtedly arises from that compound's competitive inhibition ($K_i = 8 \times 10^{-6}$ M, Table II).

TABLE III: Effect of the Time of Preexposure to Pyridoxal on the Activity of Pyridoxine Kinase.

Pyridoxal Concn (M)	Time of Preexposure to Pyridoxal (min)	Sp Act.[a] (μmoles/min per mg)
4×10^{-4}	0	1.6
4×10^{-4}	7.5	0.50
4×10^{-4}	15	0.36
4×10^{-4}	30	0.16
4×10^{-4}	60	0.10
5×10^{-5}	0	1.6
5×10^{-5}	7.5	1.4
5×10^{-5}	15	1.1
5×10^{-5}	30	0.88

[a] Measured by direct spectrophotometry at 388 nm.

TABLE IV: Comparison of the Inhibitory Effects of Three Pyridoxine Kinase Substrates.

Substrate to Which Enzyme Was Exposed	Sp Act.[a] (μmoles/min per mg)		Rate Measured as % of Control
	Preexposed	Control[b]	
5×10^{-5} pyridoxal	0.75	1.6	50
5×10^{-5} M pyridoxamine	1.6	1.75	95
5×10^{-5} M pyridoxol	0.5	0.56	91

[a] Assayed by direct spectrophotometry at 388 nm. In each case final pyridoxal concentration at start of assay was 0.4 mM. [b] Control contained all components of experimental, but they were added only at the moment assay was begun.

Discussion

From both the purification factor and the molecular weight of the kinase, the number of molecules of pyridoxine kinase per cell can be estimated if the amount of protein extractable from a cell of 1-pl volume is 7.5×10^{-14} g. (An average dry weight for an *E. coli* of 1-pl volume is then 2.5×10^{-13} g/cell (Luria, 1960). We empirically determined that about 30% of the dry weight of *E. coli* is extractable as soluble protein by ultrasonic disruption and centrifugation.) Since pyridoxine kinase has a molecular weight of approximately 40,000 and appears homogeneous after a 3000-fold purification, it probably represents $1/3000$ of the total extract protein or 360 molecules/cell.

This value of 360 molecules of kinase/cell is in the range of normal enzyme concentrations (Srere, 1967) and thus argues against McIlwain's (1946) hypothesis which states that the

enzymes involved in the biosynthesis of coenzymes may be present in concentrations of one or a few molecules per cell. The data, however, do not exclude the possibility that either one or more of the enzymes in the earlier part of the pathway are present in such low concentrations. Because the work reported here excludes a very low pyridoxine kinase enzyme concentration, the alternative hypothesis, that enzymes involved in biosynthesis of coenzymes have low turnover numbers, apparently is the correct one.

It is presumably advantageous to a cell to limit the concentration of free pyridoxal 5′-phosphate, because this highly reactive molecule has been shown to react strongly with certain ϵ-amino groups of proteins, such as albumin (Dempsey and Christensen, 1962) and phosphofructokinase (Uyeda, 1969), which have no obvious metabolic relationship to this coenzyme.

If such reactions were to occur without restriction between pyridoxal 5′-phosphate and these proteins we might find growth restricted. In other words pyridoxal 5′-phosphate might reasonably be assumed to be toxic when present in excess because of its ready reactivity toward ϵ amino groups. Accordingly we feel it is logical to expect not only that the cell has a mechanism to limit the concentration of pyridoxal 5′-phosphate obtained from *de novo* synthesis, but also that it has one or more mechanisms to prevent excessive pyridoxal 5′-phosphate concentrations when B_6 vitamers are present in the medium.

It has already been established that the biosynthesis of vitamin B_6 is controlled in *E. coli* (Dempsey, 1965) and that the effector of control is neither pyridoxol nor pyridoxol 5′-phosphate (Dempsey, 1966). The specific effector of this control is presently undetermined. When an external source of B_6 vitamers becomes available to *E. coli*, controls which normally operate on the early enzymes of the pyridoxine biosynthetic pathway would not be expected to limit the conversion of these vitamers into pyridoxal 5′-phosphate. Control of pyridoxal phosphate biosynthesis from externally supplied vitamers then must be exerted on either of the last two steps (oxidation and/or phosphorylation) or on the transport of the vitamers into the internal environment.

The finding that pyridoxal inactivates the kinase enzyme together with the possibility that *E. coli* contains a pyridoxal 5′-phosphate phosphatase (Turner and Happold, 1961) indicated the possibility for a control acting upon the latter steps of the pathway. In such a system pyridoxal 5′-phosphate biosynthesis from pyridoxol might be controlled in *E. coli* by hydrolysis of pyridoxal 5′-phosphate to pyridoxal which then inactivates the kinase enzyme. The effect of this scheme (Figure 6) would be that excess pyridoxal 5′-phosphate would be converted by the phosphatase to pyridoxal. Since pyridoxal then would increase in concentration instead of pyridoxal 5′-phosphate, the presumably toxic interaction of pyridoxal 5′-phosphate with ϵ amino groups would be avoided and at the same time its further synthesis would be diminished by the inactivation of the kinase by pyridoxal.

A mechanism whereby pyridoxal rather than pyridoxal 5′-phosphate would serve as the controlling molecule would be of advantage to the cell because pyridoxal lacks the highly reactive free aldehyde group of pyridoxal 5′-phosphate. Instead, pyridoxal has its aldehyde group bound in a relatively inactive hemiacetal (Metzler and Snell, 1955).

The $K_{m,app}$ determinations with the coupled ADP assay

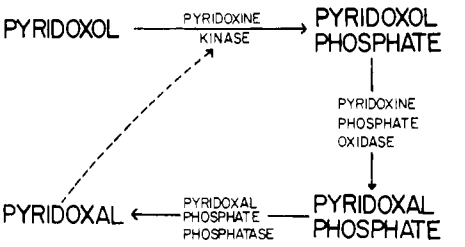

FIGURE 6: A scheme of pyridoxal 5′-phosphate metabolism in *E. coli*.

(Table II) substantiate the implications of the $K_{i,app}$ data obtained by inhibition studies of pyridoxal phosphorylation. These $K_{m,app}$ and K_i values, the maximum velocity values, together with the data concerning the extents of phosphorylation at low substrate concentrations (Kenny and Dempsey, 1967) indicate that pyridoxol is more likely to be the natural substrate of the kinase than is pyridoxal.

If pyridoxol is the natural biosynthetic substrate, then the conversion of pyridoxol into pyridoxal 5′-phosphate would occur by phosphorylation of pyridoxol followed by oxidation of the pyridoxol 5′-phosphate to pyridoxal 5′-phosphate. Consistent with this hypothesis was the previous finding (Dempsey, 1966) that a pyridoxal auxotroph lacking pyridoxol phosphate oxidase accumulated both pyridoxol and pyridoxol 5′-phosphate when starved for pyridoxal. These data, together with Henderson's demonstration of pyridoxol 5′-phosphate oxidase activity in microorganisms (1965), strongly suggest that the sequence of the last two steps is indeed phosphorylation followed by oxidation.

Confirmation of pyridoxol as the natural biosynthetic substrate, however, awaits the elucidation of the entire pathway for pyridoxal 5′-phosphate biosynthesis in *E. coli* B. Only this information can unequivocally confirm that phosphorylation does not occur at an earlier step prior to pyridoxine biosynthesis. If this latter possibility were the case, the pyridoxine kinase enzyme would not be part of the biosynthetic pathway; instead, the enzyme might serve some sort of salvage function in the cell.

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Reaction of Glyceraldehyde 3-Phosphate Dehydrogenase with Aliphatic Aldehydes*

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ABSTRACT: The reaction of glyceraldehyde 3-phosphate dehydrogenase with a series of aliphatic aldehydes has been studied at 25°. A plot of log V_{max} *vs.* σ^*, the Taft substituent constant, is linear with a slope of 2.08. Thus the rate of the reaction is facilitated by electron withdrawing substituents. Steric factors are of minor importance. Increased steric bulk in the aldehyde did not in general produce significant deviations in the plot of log V_{max} *vs.* σ^*, although there was positive deviation of the points for isovaleraldehyde and isobutyraldehyde. Arsenate had no effect on the rate of the reactions. Trimethylacetyl phosphate is an inhibitor toward these substrates. This inhibition is of the noncompetitive type. Normal inhibition kinetics are observed, plots of $1/V$ *vs.* [I] being linear rather than sigmoidal as is the case when the natural substrate glyceraldehyde 3-phosphate is employed.

D-Glyceraldehyde 3-phosphate dehydrogenase (D-glyceraldehyde 3-phosphate:NAD oxidoreductase (phosphorylating), EC 1.2.1.12) is a key enzyme of carbohydrate metabolism, catalyzing several different reactions depending on the reaction conditions (Colowick *et al.*, 1966). The normal dehydrogenase reaction in the presence of P_i involves the conversion of glyceraldehyde 3-phosphate into 1,3-diphosphoglyceric acid. NAD^+ is required as a cofactor and in the reaction is converted into NADH. In addition, an acyl phosphatase activity has been noted in the presence of NAD^+ (Harting and Velick, 1954; Park and Koshland, 1958; Malhotra and Bernhard, 1968; Phillips and Fife, 1969), and esterase activity has been detected toward phenolic esters with an enzyme from which NAD^+ has been removed (Park *et al.*, 1961). The same thiol ester intermediate is apparently formed in reaction of the enzyme with acetyl phosphate and *p*-nitrophenyl acetate (Mathew *et al.*, 1967). Mechanisms have been suggested for action of the enzyme (Olson and Park, 1964), the evidence pointing strongly to involvement of a thiol group and more ambiguous evidence implicating the imidazole ring of histidine (Halcomb *et al.*, 1968; Friedrich *et al.*, 1964). However, conclusive mechanistic evidence is lacking.

The study of steric effects in α-chymotrypsin-catalyzed reactions has given results that could be directly related to the mechanism of the deacylation reaction (Fife and Milstien, 1967; Milstien and Fife, 1968). Steric effects were also studied in the acyl phosphatase reaction catalyzed by glyceraldehyde 3-phosphate dehydrogenase (Phillips and Fife, 1969), and it was found that branching in the acyl group has a profound influence on the reaction. Also, trimethylacetyl phosphate, although not a substrate, was an excellent inhibitor for both the acetyl phosphate activity and the dehydrogenase reaction involving glyceraldehyde 3-phosphate. For the latter reaction, plots of $1/V$ *vs.* [I] were sigmoidal. In continuing studies of steric effects in reactions catalyzed by this enzyme we have now employed a series of variously substituted aliphatic aldehydes as substrates to obtain information concerning the mechanism of the dehydrogenase reaction and to further ascertain

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