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EVIDENCE FOR THE FORMATION OF
PYRIDOXIMINE-5-PHOSPHATE BY THE BIODEGRADATIVE
L-THREONINE DEHYDRASE OF ESCHERICHIA COLI

presented by

John Frank Riebow

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W. A. Wood
Major professor

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EVIDENCE FOR THE FORMATION OF PYRIDOXIMINE-5-PHOSPHATE BY THE BIODEGRADATIVE L-THREONINE DEHYDRASE OF *ESCHERICHIA COLI*

By

John Frank Riebow

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ABSTRACT

EVIDENCE FOR THE FORMATION OF PYRIDOXIMINE-5-PHOSPHATE BY THE BIODEGRADATIVE L-THREONINE DEHYDRASE OF ESCHERICHIA COLI

By

John Frank Riebow

Until recently, the α,β-elimination reactions of various amino acids, which are catalyzed by pyridoxal phosphate dependent enzymes, were considered to be irreversible. Snell and Watanabe have shown that the tryptophanase catalyzed α,β-elimination reaction is reversible (Watanabe, I., and Snell, E. E., *Pro. Nat. Acad. Sci. USA 69*, 1086 (1972). In addition, it was established that the mechanism by which the reverse reaction proceeded was different from the mechanism for the forward reaction. One intermediate for the reverse reaction was established as pyridoximine phosphate.

This research project was undertaken to determine if the reversibility of the α,β-elimination reaction catalyzed by tryptophanase is unique to this enzyme; or, if the biodegradative L-threonine dehydrase from *E. coli*, another pyridoxal phosphate dependent enzyme, would also catalyze the reverse of its reaction by a mechanism similar to that for tryptophanase.

John Frank Riebow

When threonine dehydrase was incubated in the presence of high levels of ammonium chloride, an intermediate was formed which has been indirectly shown to be pyridoximine phosphate.

To my parents

ACKNOWLEDGEMENTS

The author wishes to extend his sincere appreciation to Dr. W. A. Wood for his encouragement and support throughout the course of this research. The many discussions and friendship of Mr. David Le Blond are also recognized. I would like to extend a special thanks to Mrs. Jane Fortman for her expertise in the typing of this manuscript. Finally, I would like to acknowledge some very dear friends of mine whose Christian fellowship and encouragement is forever appreciated: Greg Allen, Dick and Beverly Anderson, Gilbert and Jane Apps, Doris Bauer, Charley and Lydia Brown, Martha Burley, Ed and Pat Comandella, Helen Dorriell, Sheila Fairman, Al Getzen, Sandy Getzen, Jeff and Sally Harrold, Dave and Peggy Mingus, Herman and Henrietta Prether, Eric and Cheryl Satterlee, Don and Dorothy Snider, Minnie and Kim Worman.

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INTRODUCTION

For some time, α,β-elimination reactions, which are catalyzed by a variety of pyridoxal phosphate-dependent enzymes, have been considered to proceed by way of a common mechanism. This generalized mechanism can be summarized in the following manner (see figure 4 for the various structures).

$$\begin{tikzcd}
\alpha\text{-amino acid} + \text{PLP-enzyme} \arrow[r] & [\text{enzyme-PLP-intermediate}]_3 \arrow[d] \\
& [\text{enzyme-PLP-}\alpha,\beta\text{-unsaturated amino acid}] \arrow[d] \\
& \text{PLP-enzyme} + \alpha,\beta\text{-unsaturated amino acid} \arrow[d] \\
\alpha\text{-keto acid} + NH_3 & \alpha\text{-imino acid} \arrow[l, "H_2O"]
\end{tikzcd}$$

Experimental evidence in support of this mechanism has been provided from both isotope incorporation and spectral experiments.

Snell and his associates have recently demonstrated that the α,β-elimination reaction, as catalyzed by tryptophanase, proceeds by way of a mechanism different from the one shown above in the terminal steps of the reaction. This new alternative pathway involves the tautomerism of the α-aminoacrylate pyridoxal phosphate Schiff base (enamine) to the corresponding ketamine, which is then hydrated to the carbinolamine. As a result of an elimination, free pyruvate is formed along with enzyme bound pyridoximine phosphate. Hydrolysis of the latter enzyme intermediate results in the regeneration of the pyridoxal phosphate form of the enzyme with the release of ammonia. As a result of this work by Snell's group, there is now a need to determine if this new alternative mechanism is unique to tryptophanase, or if there are other pyridoxal phosphate enzymes which function by a similar mechanism.

The work, presented here, is an attempt to establish whether or not an appreciable amount of enzyme bound pyridoximine phosphate is formed as a result of the interaction between high levels of ammonia and threonine dehydrase. The enzyme directed formation of pyridoximine phosphate, under the above conditions, would be evidence in support of the mechanism proposed by Snell, and that this mechanism is not unique to tryptophanase.

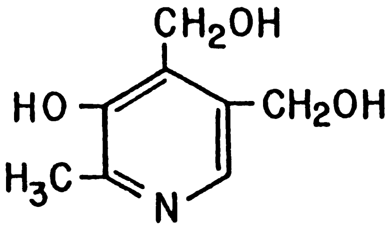
LITERATURE REVIEW

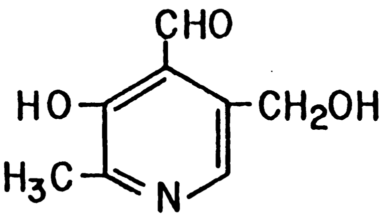
Since the discovery of vitamin B_6 during the 1930's, a great deal of research effort has been directed toward establishing its involvement in metabolism and elucidating its function at the molecular level. Several detailed reviews on various aspects of vitamin B_6 have appeared over the past twenty years (1-4). These reviews examine in detail, the many reaction types in which vitamin B_6 plays a role in catalysis. The following general discussion is intended to summarize one of the classes of reactions for which vitamin B_6 is known to play a functional role, the α,β-elimination reaction.

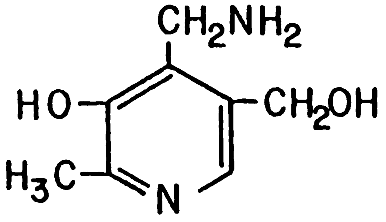
Discovery of the Role of Pyridoxal Phosphate in Metabolism

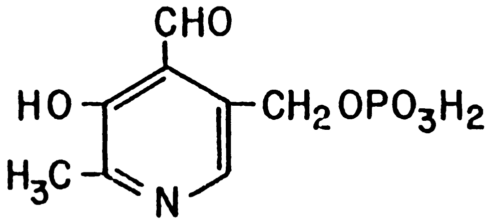
The involvement of vitamin B_6 (see figure 1) in protein metabolism became evident during the mid 1940's. Earlier work by Gyorgy had demonstrated that a nutritional factor, termed vitamin B_6, would cure a specific dermatitis of young rats which were fed a vitamin-deficient diet. In 1936, Birch and Gyorgy partially characterized vitamin B_6 chemically. Crystalline pyridoxol from various natural sources was prepared in several laboratories (5-7) during 1938, and was subsequently synthesized in other laboratories (8).

Figure 1. Vitamin B_6 structures. Listed here are the various forms of vitamin B_6. The term pyridoxine has, in the past, been used interchangably with vitamin B_6. Currently, the term pyridoxine is used interchangably with pyridoxol.


Pyridoxol


Pyridoxal


Pyridoxamine


Pyridoxal-5-Phosphate

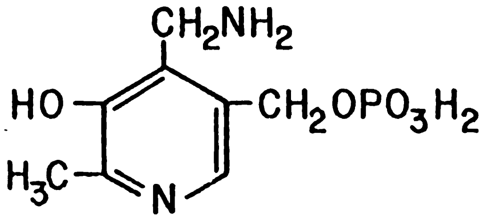

Pyridoxamine-5-Phosphate

Figure 1

Following the isolation of crystalline pyridoxol, several nutritional studies during the early 1940's established a requirement for pyridoxol in protein metabolism. Experiments involving vitamin B_6-deficient animals revealed that protein metabolism was impaired under these conditions. It was also shown that high protein diets caused an increase in the rate of appearance of vitamin deficiency in these animals. The toxic effects which are caused by high intake of certain amino acids (serine, glycine, and methionine in particular) in vitamin B_6-deficient animals could be controlled by administering pyridoxol to these animals. In addition to these mammalian systems, the biosynthesis of alanine, threonine, lysine, and other amino acids was shown to be inhibited in certain strains of bacteria (lactic acid bacteria and *Clostridium welchii*) when grown on vitamin B_6-deficient media [see (2) and references therein].

During this same period of time, Snell (9) established that other forms related to pyridoxol, which were obtainable from natural sources, were much more effective in stimulating the growth of lactic acid bacteria. Subsequently, Snell demonstrated that these pyridoxol derivatives were pyridoxal and pyridoxamine. In addition, it was found that pyridoxal and pyridoxamine account for the majority of the vitamin B_6 which is found in natural substances.

Additional work by Snell (10) demonstrated that pyridoxamine could be formed by a nonenzymatic reaction between pyridoxal and various amino acids. This led to the prediction that the interconversion between pyridoxal and pyridoxamine might occur *in vivo* during transamination between various amino acids as is shown below.

$$\text{amino acid}_1 + \text{pyridoxal} \rightleftharpoons \text{keto acid}_2 + \text{pyridoxamine}$$

$$\text{keto acid}_1 + \text{pyridoxamine} \rightleftharpoons \text{amino acid}_2 + \text{pyridoxal}$$

$$\overline{\text{amino acid}_1 + \text{keto acid}_1 \rightleftharpoons \text{amino acid}_2 + \text{keto acid}_2}$$

Concurrent work in other laboratories on various enzymes associated with amino acid metabolism was beginning to suggest a functional role for vitamin B_6. Various studies on bacterial systems had revealed that vitamin B_6 was necessary for the decarboxylation of various amino acids (11). In addition, it was demonstrated that the actual form of vitamin B_6 required for decarboxylation was pyridoxal. Further work on purified apo-enzymes of amino acid metabolism, in particular enzymes involved in transamination reactions, provided direct evidence that pyridoxal-5-phosphate was the active form of the coenzyme (12-14).

Pyridoxal Catalyzed Nonenzymatic Reactions

As mentioned above, pyridoxal will catalyze nonenzymatic transamination reactions. Metzler and Snell were able to take advantage of this system to evaluate the importance of each substituent of pyridoxal towards the rate of ketoglutarate formation from glutamate [see (1) and references therein].

Figure 2 gives some of the model compounds which Metzler and Snell employed in their studies.

The results of these studies indicate that the heterocyclic nitrogen atom, the phenolic hydroxyl group at position 3, and the formyl group at position 4 are all essential for nonenzymatic catalysis. The methyl group at position 2 may contribute to the catalytic efficiency of pyridoxal, through inductive effects, but its presence is not an absolute requirement for catalysis. The hydroxymethyl group at position 5 is not required for nonenzymatic catalysis; although, it is essential for the functioning of pyridoxal biologically since it is this group which is phospharylated to give pyridoxal-5-phosphate. While evaluating the importance of the hydroxymethyl group, Metzler *et al.* (15) found that either the absence of this group, or its phosphorylated form, was actually more efficient catalytically than was pyridoxal. They suggested that these results indicated that, under the experimental conditions used, the aldehyde group of pyridoxal is in equilibrium with its hemiacetal form. Hemiacetal formation, in effect, lowers the concentration of free aldehyde necessary for catalysis.

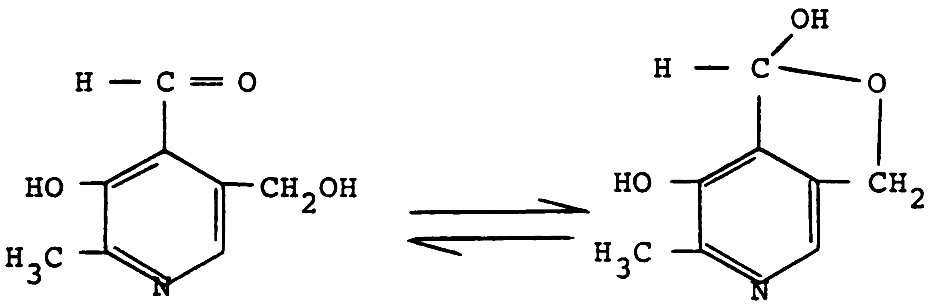
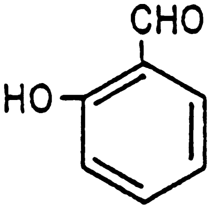
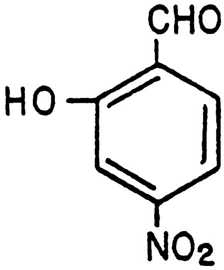


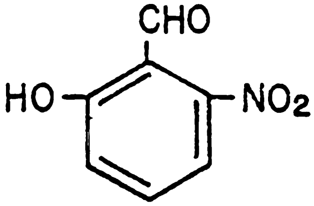
Figure 2. The structure of salicylaldehyde derivatives related to pyridoxol. The various derivatives of salicylaldehyde shown were used by Metzler and Snell to establish the structure-function relationship of the various substituents of pyridoxal in nonenzymatic catalysis.



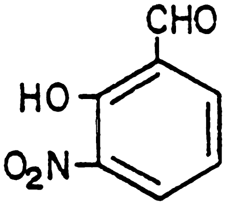
Salicylaldehyde



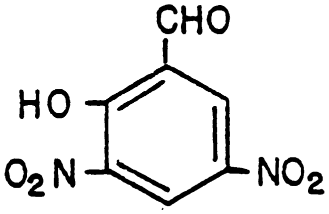
4-Nitrosalicylaldehyde



6-Nitrosalicylaldehyde



3-Nitrosalicylaldehyde

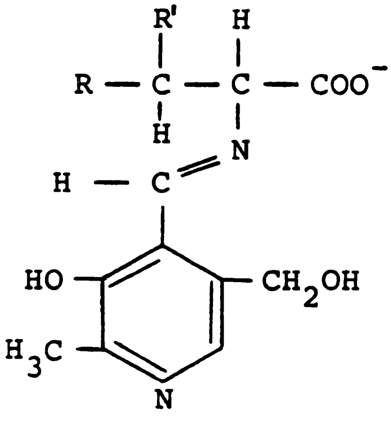


3,5-Dinitrosalicylaldehyde

Figure 2

It has been known for some time that reactions between amines and aldehydes lead to the formation of imine (Schiff's base) compounds. By analogy, the yellow color, which appears when various amino acids are mixed with pyridoxal (or pyridoxal phosphate), can be attributed to imine formation between the amino group of the amino acid and the free aldehyde group of pyridoxal. These imine compounds have a characteristic absorption maximum in the region from about 340 to 440 nm. The exact location of the absorption maximum is dependent upon the particular amino acid and the pH of the solution. Experimental evidence obtained by Metzler (16) and Christensen (17) demonstrated that, under physiological conditions, a significant amount of pyridoximines are formed during the reaction between pyridoxal and various amino acids. The existence of such pyridoximines had been postulated by several investigators prior to the above publications.

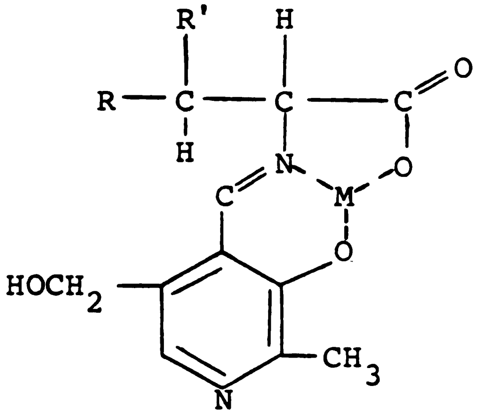
As a result of imine formation between an amino acid and pyridoxal, a structure is formed in which there is a



strong withdrawal of electrons from the α-carbon atom of the amino acid toward the heterocyclic nitrogen atom; thus weakening the various bonds to the α-carbon atom.

Support for this concept of charge delocalization, as a result of imine formation, was provided by the demonstration that 4-nitrosalicylaldehyde and 6-nitrosalicylaldehyde were effective catalysts for nonenzymatic transamination. For these compounds, electron withdrawal from the α-carbon atom (for the corresponding imines to the ones shown above), through resonance structures, can be demonstrated. The compounds 5-nitrosalicylaldehyde and 3,5-dinitrosalicylaldehyde will not catalyze nonenzymatic transamination of amino acids. For these compounds, the position of the nitro group(s) does not allow for electron delocalization through resonance structures. These results indicated that the positioning of the strongly electronegative nitro group dictated the catalytic efficiency of various nitrosalicylaldehyde compounds (see figure 2 for structures).

The phenolic group of pyridoxal is important due to the fact that, through hydrogen bonding, it can stabilize the imine which is formed between an amino acid and the aldehyde group of pyridoxal. It has been known for some time that the rate of nonenzymatic transamination of amino acids, catalyzed by pyridoxal, was greatly enhanced by various metal ions. This rate enhancement has been explained in terms of chelated ring structures which involve the phenolic group of pyridoxal. The chelated ring structures add stability to the imine structure and also force this entire system of double bonds into a planar



configuration that allows for maximal electron withdrawal from the α-carbon atom by the heterocyclic nitrogen atom.

These various studies on the nonenzymatic transamination by pyridoxal, and structurally related compounds, allowed Metzler *et al.* (18) to propose a general mechanism for pyridoxal catalysis in 1954. This mechanism could account for all of the known reactions of amino acids in which pyridoxal (or pyridoxal phosphate) was a catalyst. These reactions included racemization, transamination, decarboxylation, α,β-elimination, β,γ-elimination, and β-substitution reactions. All of these reactions proceed through a common imine intermediate and are subsequently the result of specific bond cleavage of the imine intermediate. Just prior to this time, Braunstein and Shamyakin (19) proposed a very similar mechanism for enzymatic reactions in which pyridoxal phosphate is a coenzyme. Their mechanism was based on theoretical considerations only.

A Mechanism for Pyridoxal Catalyzed α,β-Elimination Reactions

In the following discussion, only the mechanism for α,β-elimination will be considered. This mechanism is shown in figure 3 for a nonenzyme catalyzed reaction.

Initially pyridoxal and α-amino acid combine to form an imine structure (structure I in figure 3), which is stabilized by the presence of a metal ion (M). As a result of the strong withdrawal of electrons from the α-carbon of the amino acid toward the heterocyclic nitrogen atom, the proton on the α-carbon atom dissociates to give structure II (figure 3). Rearrangement of this conjugated double bond system results in the release of the R' group, as an anion, from the β-carbon atom of the amino acid. The resulting α,β-unsaturated imine (structure III in figure 3) then decomposes, in aqueous media, through an α,β-unsaturated amino acid, to yield the corresponding β-keto acid and free ammonia.

Several pyridoxal phosphate-dependent enzymes are known which catalyze α,β-elimination reactions of amino acids by a similar mechanism (19). There are some important differences between nonenzymatic and the corresponding enzymatic reactions. Enzymatic reactions, for which pyridoxal phosphate is a coenzyme, proceed at a rate much greater than for the corresponding nonenzymatic reaction. The enzymatic reaction is highly specific, each enzyme catalyzes one reaction. In nonenzymatic systems, several reactions occur simultaneously with

Figure 3. Mechanism for the nonenzymatic α,β-elimination reaction of Metzler *et al.* (18) of amino acids as catalyzed by pyridoxal.

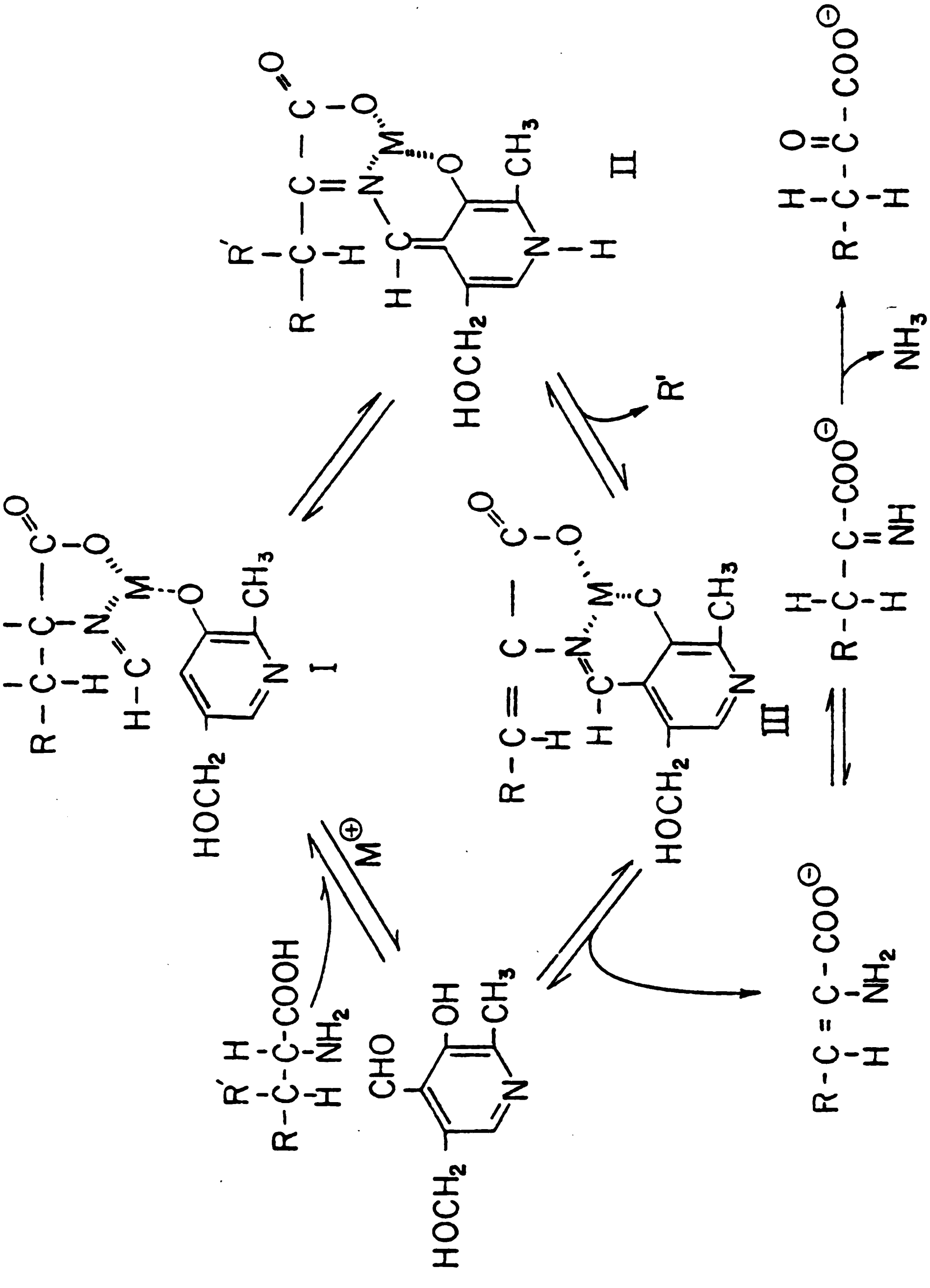


Figure 3

little specificity. This substrate specificity and rate enhancement, present in enzymatic systems, can only be attributed to the protein moiety. Table I lists some enzymes which catalyze α,β-elimination reactions.

The currently accepted mechanism for pyridoxal phosphate-mediated α,β-elimination reactions is shown in figure 4 (structures I, II, III, IV, V, and VI). The individual steps of this mechanism will be discussed in terms of the experimental evidence which supports their involvement. Recently, Snell and his associates (20) presented evidence for an alternative mechanism for the terminal steps of the reaction. This alternative mechanism is also shown in figure 4 (structures I, II, III, IV, V, VII, and VIII).

For all enzymes, in which pyridoxal phosphate is a coenzyme, the pyridoxal phosphate group is covalently bound, as an imine, to a lysyl residue of the enzyme (structure I in figure 4). This imine can be reduced with sodium borohydride and the resulting N^6-pyridoxyl-lysine residue can be isolated following proteolytic digestion of the enzyme (21). In addition, as a result of the imine, all pyridoxal phosphate-dependent enzymes exhibit a characteristic absorption spectra in the region of 410 to 430 nm (21-25). The appearance of an absorption peak in this region is consistent with the theoretically predicted location for the imine form of pyridoxal phosphate.

Structure II represents the binding of substrate to the enzyme (formation of a Michaelis complex). In general,

Table 1. Pyridoxal phosphate-dependent enzymes which catalyze α,β-elimination reactions of various amino acids.

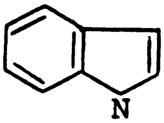
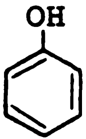
enzyme	R	R'
L-Threonine Dehydrase	CH_3	OH
D-Serine Dehydrase	H	OH
Tryptophanase	H	 indole
β-Tyrosinase	H	 phenol
Cystiene Sulfhydrase	H	SH

Figure 4. Generalized mechanism for the α,β-elimination reaction of amino acids as catalyzed by pyridoxal phosphate-dependent enzymes. The currently accepted general mechanism is shown by structures I, II, III, IV, V, and VI. An alternative pathway for tryptophanase has been proposed Watanage and Snell (20), structures I, II, III, IV, V, VII, and VIII.

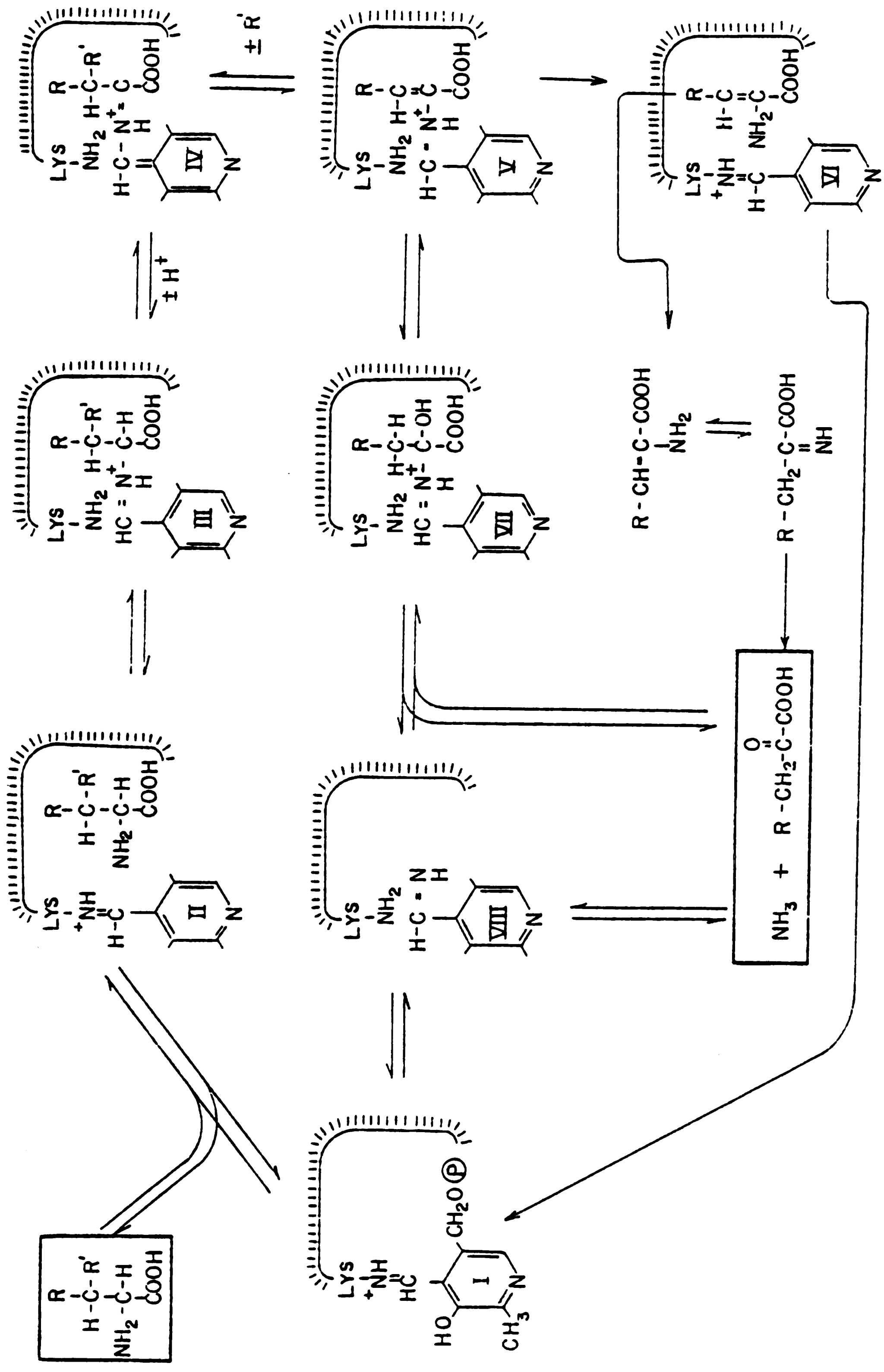

I
II
III
IV
V
VI
VII
VIII
LYS
± H+
± R'
R-CH-C-COOH
NH2
R-CH2-C-COOH
NH
NH3 + R-CH2-C-COOH
O
CH2O(P)
HO
CH3
R
H-C-R'
NH2-C-H
COOH

Figure 4

the formation of this type of a complex can be considered a necessary prerequisite for catalysis. Evidence that a Schiff's base is formed between the enzyme bound pyridoxal phosphate moiety and the substrate for the enzyme (structure III) comes from absorption and circular dichroism spectra for various pyridoxal phosphate enzymes in the presence of their respective substrates (4). The use of various inhibitors (both competitive and noncompetitive) has been quite helpful in these types of studies.

Evidence for structure IV is largely the result of spectral observations of pyridoxal phosphate enzymes in the presence of their substrates. When the enzyme is incubated with its substrate, there is a shift in the absorption maximum from around 410 to 430 nm. to near 500 nm. (the exact location of these peaks is dependent upon the particular enzyme). The position and the shape of the latter absorption maximum is more characteristic of quinonoid structures (structure IV in figure 4) than for aromatic structures. These longer wavelength peaks are transient and disappear as the substrate is consumed in the reaction (see reference 4).

The existence of structure V has been established on the basis of an observed absorption difference peak in the region of 450 to 470 nm. Structures such as V, are theoretically predicted to absorb in this vicinity. Also, these peaks are dependent upon the presence of substrate and disappear as the substrate is removed.

The conversion of structure V to structure I with the corresponding enamine remaining bound to the enzyme is supported by the work of Flavin and Slaughter (26). These investigators were able to demonstrate that the addition of N-ethylmaleimide to reactive enamine intermediates, similar to structure V, results in the stereospecific formation of the product α-keto-β-[3'-(N-ethyl-2',3'-dioxopyrrolidyl)] butyrate. When the threonine dehydrase reaction is run in deuterium oxide (D_2O), deuterium is placed on the β-carbon atom of the product of α-ketobutyrate. In addition, if sodium borohydride is added to the reaction mixture, D,L-α-aminobutyrate can be isolated. These two experiments taken together indicate that free α-aminocrotonate is released from the enzyme and that it is spontaneously converted to a α-ketobutyrate and ammonia via α-iminobutyrate

Wood and Phillips (21) provided some additional information concerning this mechanism. From isotope incorporation experiments, it was demonstrated that deuterium and ^{18}O from the aqueous medium are back incorporated into threonine by threonine dehydrase. These results indicated that the reactions leading from structure II through structure V are freely reversible and that, at least for threonine dehydrase, the rate limiting step in the mechanism occurred somewhere after structure V.

As mentioned above, Snell and his associates (20) have established an alternative pathway for the terminal steps of the mechanism shown in figure 4 (structures I, II, III, IV, V, VII, and VIII).

This alternative mechanism became evident from experimental data for the enzymatic synthesis of tryptophan from indole, pyruvate, and ammonia as catalyzed by tryptophanase. From a series of kinetic experiments, Watana-e and Snell (20) were able to develop a kinetic mechanism for the reverse of the tryptophanase reaction. This mechanism was shown to be consistent with the ordered Ter-Uni mechanism of Cleland (27). This mechanism indicated that pyruvate was the second substrate to bind to the enzyme with indole or ammonia being the first substrate to bind. It was not possible, from the kinetic data alone, to demonstrate whether indole or ammonia was the first substrate to bind to the enzyme.

Holotryptophanase has a characteristic absorption maximum at 337 nm. In the presence of 1 M ammonium chloride, this peak is intensified considerably and is shifted to near 420 nm. Free pyridoxal phosphate also shows a similar red shift in the presence of high levels of ammonia (from about 385 to 405 nm.)(20). Reduction of the incubation mixture of pyridoxal phosphate and ammonium chloride, with sodium borohydride, resulted in the formation of pyridoxamine phosphate. From these spectral studies, Snell and his associates concluded that the shift in the absorption maximum of tryptophanase to 420 nm., in the presence of ammonium chloride, was the result of the formation of an enzyme-bound pyridoximine phosphate complex. The results of these spectral studies, along with the kinetic experiments allowed Snell's group to conclude that in the formation of tryptophan, by tryptophanase, ammonia was the first substrate to bind to the enzyme, then pyruvate, and finally indole.

METHODS AND MATERIALS

Bacteriological

An isoleucine requiring mutant of *Escherichia coli* (ATCC 9739, designated LA-9) was maintained on a nutrient agar slant at 4°C. Large quantities of the organism were prepared by transferring cells from the nutrient slant into 10 ml. of nutrient broth. The organism was allowed to grow for 12 hours at 37°C., with mechanical shaking. The incubating solution was then transferred to a flask containing 1 ℓ. of complex medium [2% (w/v) N-Z amine NAK (an enzymatic digest of casein), 1% (w/v) yeast extract, and 0.5% (w/v) potassium phosphate (dibasic)]. The organism was allowed to grow at 37°C. for 12 hours (without shaking). Following this, the organism was transferred to 100 ℓ. of the same complex medium and were grown for 4 hours at 37°C. The cells were then harvested and stored at -20°C. until needed for preparation of crude extract.

Chemicals

Glutamate-oxalacetate transaminase (holoenzyme, GOT) and malate dehydrogenase were purchased from Boehinger Mannheim Biochemicals. Lactate dehydrogenase (beef heart, type III), D,L-dithiothreitol, pyridoxol-5-phosphate, pyridoxamine-5-phosphate, and adenosine-5'-monophosphate were purchased from Sigma Chemical Company. Nicotinamide adenosine dinucleotide reduced disodium salt (NADH) was obtained from P-L Biochemicals, Inc. L-Glutamic acid and α-ketoglutarate sodium salt (both A grade) were purchased from Calbiochem. L-Aspartic acid and yeast extract were obtained from General Biochemicals. N-Z Amine NAK was obtained from Sheffield Chemical Co. Nutrient agar and nutrient broth were purchased from Difco Laboratories. All other chemicals were purchased from commercial suppliers. Reagent or analytical grades were always used.

Determinations and Procedures

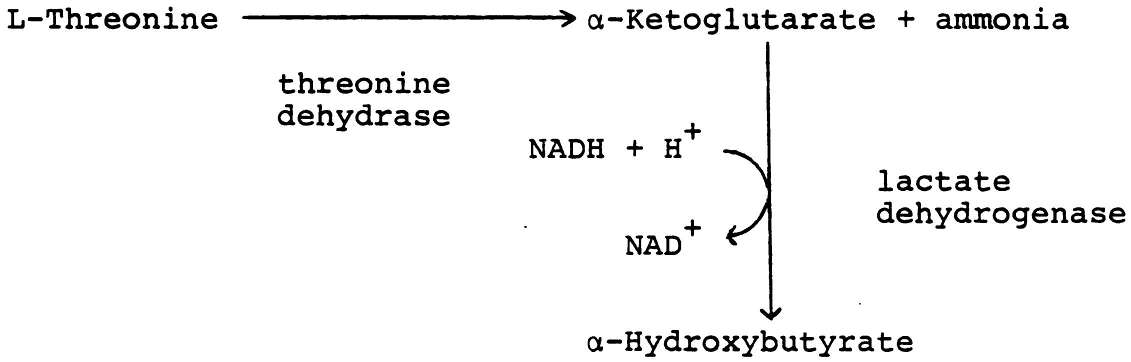
Protein Determinations

In most instances, protein concentration was estimated by the method of Lowry *et al.* (28). The protein samples (approximately 100 μg) and bovine serum albumin standards (20 to 200 μg) were diluted to 0.5 ml. with water. These samples were then further diluted by the addition of 5.0 ml. of a solution containing 0.01% (w/v) copper sulfate, 0.02% (w/v) sodium potassium tartrate, and 3% (w/v) sodium carbonate in 0.1 N sodium hydroxide. After standing for 20 minutes at room temperature, 0.5 ml. of a 1 N phenol solution was added to each sample. These samples were incubated for 10 minutes at room temperature and then the absorbance at 750 nm. was measured for each sample. This method was always used to quantitate protein levels.

The fluorescamine assay for proteins (29) was used to monitor protein present in column effluents. Under these conditions, protein bands from columns were determined in a relative manner and no attempt was made to quantitate the protein. Aliquots (50 μℓ) from various column fractions were diluted with 1.25 ml. of sodium borate buffer (0.2 M, pH 9.0). Fluorescamine reagent (0.35 ml. of 0.30 mg/ml. fluorescamine in acetonitrile) was added to each sample with concurrent mixing with a Vortex mixer. The sample with the greatest fluorescence was used to adjust the fluorimeter to 90% of full scale deflection and the fluorescence of all other samples were measured relative to this most fluorescent sample. Figure 5 shows a typical standard curve for the Lowry protein assay and demonstrates that the fluorescamine assay is linear with respect to protein concentration.

Activity Assay for Threonine Dehydrase

Threonine dehydrase activity was measured by the lactate dehydrogenase-coupled spectrophotometric method of Dunne *et al.* (30). All assays were performed in the presence of 75 mM potassium phosphate buffer, pH 8.0, 20 mM L-threonine, 5 mM dithiothreitol, 5 mM AMP, 0.4 mM NADH, and 26 units of lactate dehydrogenase as the coupling enzyme. The final volume of the assay was 0.20 ml.



Activity Assay for Glutamate-Oxalacetate Transaminase

The enzymatic activity of glutamate-oxalacetate transaminase was measured spectrophotometrically using an enzyme-coupled assay as shown in figure 6 (31). The velocity of the transaminase was calculated from the continuous decrease in absorbance at 340 nm. as a result of NADH utilization during the course of the assay.

All assays were performed in quartz microcuvettes (0.5 ml. capacity) with a 1.0 cm. light path. A beckman Du monochromator equipped with a Gilford Model 2000 Automatic Recording Spectrophotometer was used for measuring the change in absorbance with time. All enzymatic assays were performed at 28°C. Each cuvette contained 92 mM potassium phosphate buffer (pH 7.4), 184 mM aspartate, 20.2 mM α-ketoglutarate, 0.51 mM NADH, and 1.9 units of malate dehydrogenase. The reaction was initiated by the addition of 5 μℓ. of the transaminase. The final volume in the cuvette was 0.25 ml. Figure 7 demonstrates that, under the conditions used, the assay is linear with respect to the transaminase concentration.

Figure 5. Standard curves for the Lowry (●—●) and the fluorescamine (○-○) assays for protein.

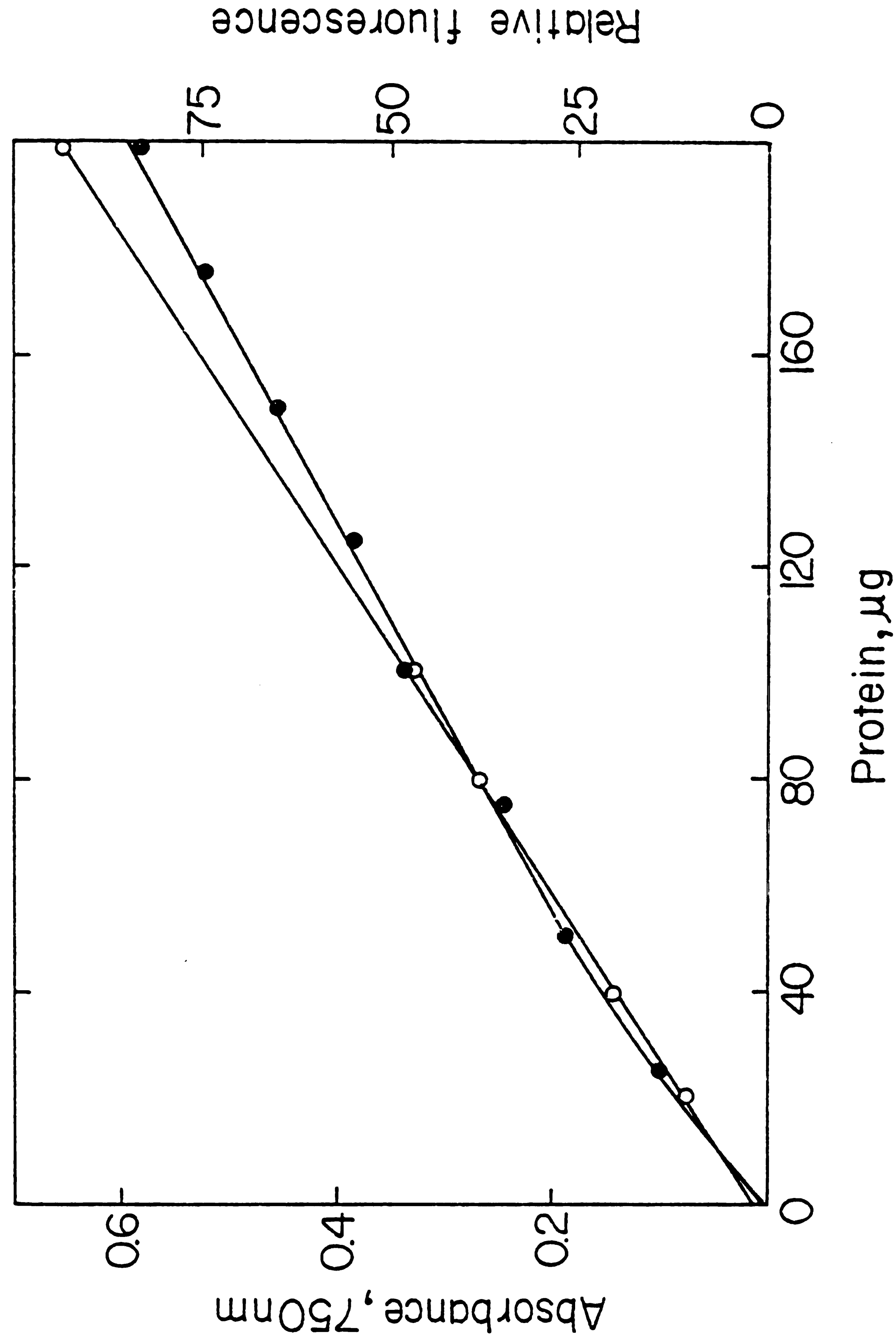

Relative fluorescence
0
25
50
75
Absorbance, 750nm
0.6
0.4
0.2
0
Protein, μg
0
40
80
120
160

Figure 5

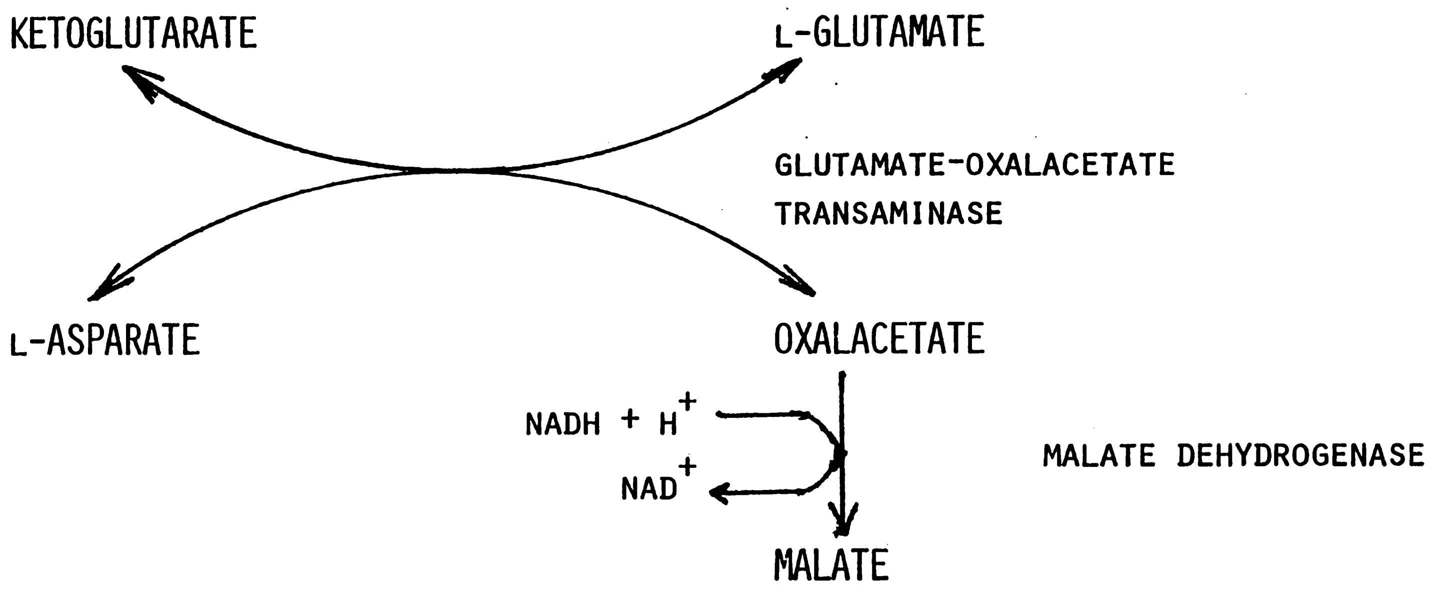


Figure 6. Activity assay for glutamate-oxalacetate transaminase

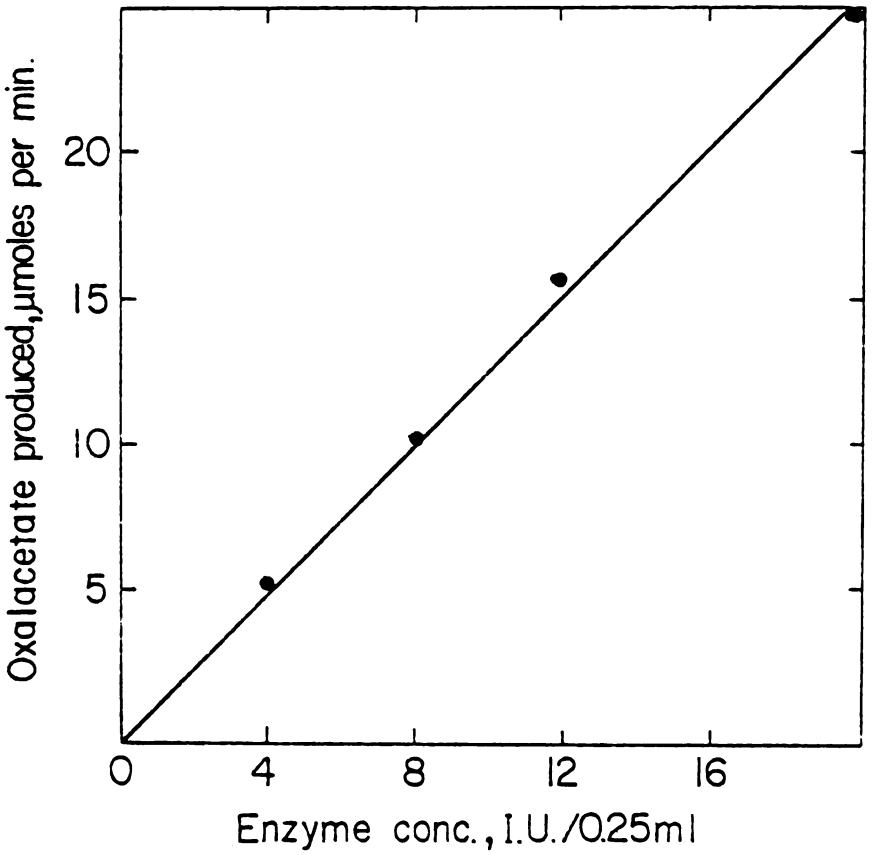


Figure 7. Linearity of the glutamate-oxalacetate transaminase assay.

Resolution of Glutamate-Oxalacetate Transaminase

A modification of the method of Wade and Snell (32) was used to prepare apo-glutamate-oxalacetate transaminase (apo-GOT). A stock solution of commercially obtained holoenzyme (10 mg/ml. in 3.2 M ammonium sulfate) was centrifuged at 18,000 rpm for 30 minutes. The precipitate (GOT) was suspended in 0.35 ml. glutamate buffer (0.5 M, pH 8.3) and allowed to stand on ice for 10 minutes. To this solution, 0.35 ml. of acetate buffer (1.0 M, pH 4.75) was added and the solution was incubated at 37°C. for 30 minutes. The solution was then made 3.2 M in ammonium sulfate sulfate [by the addition of solid $(NH_4)_2SO_4$, 0.54 mg/ml.] and the protein was collected by centrifugation at 18,000 rpm for 30 minutes. The precipitate was resuspended in 1.0 ml. potassium phosphate buffer (0.1 M, pH 7.4) and assayed as described previously. This procedure was repeated three additional times thus yielding resolved enzyme (apo-GOT). The resolved enzyme had less than 1% of its original activity. The resolution is shown in figure 8.

Activation of apo-Glutamate-Oxalacetate Transaminase with Pyridoxamine-5-Phosphate

The apo-enzyme was converted back into the holoenzyme by incubation in the presence of pyridoxamine-5-phosphate. Routinely, the enzyme was diluted 100-fold, in the presence of pyridoxamine-5-phosphate (0.5 mM, pH 7.4), and allowed to incubate at 37°C. for 30 minutes. The enzymatic activity

Figure 8. Resolution of glutamate-oxalacetate transaminase (●—●) and the ability of pyridoxamine-5-phosphate to reactivate resolved transaminase (○—○).

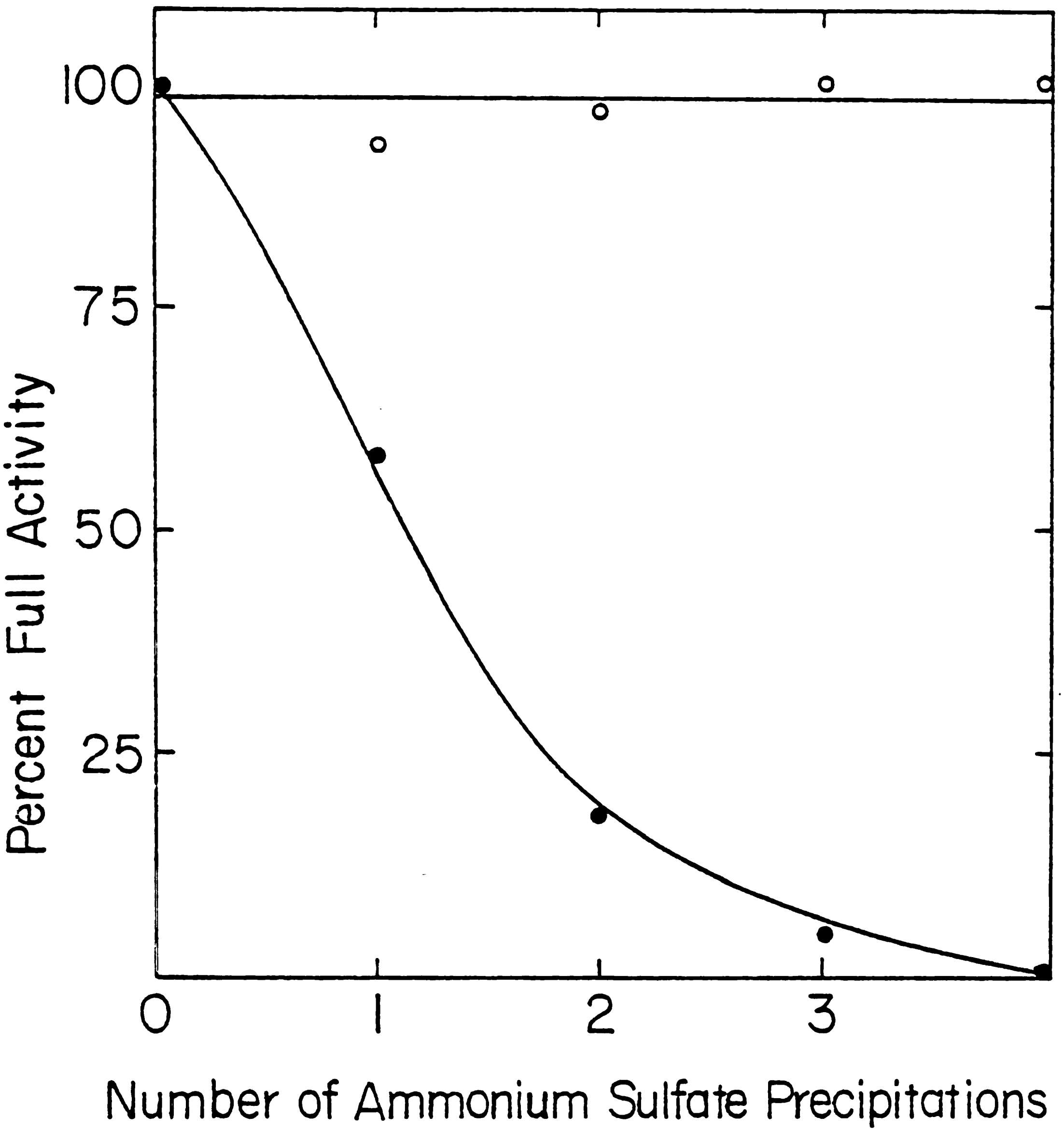

100
75
50
25
Percent Full Activity
0
1
2
3
Number of Ammonium Sulfate Precipitations

Figure 8

was then measured, as described earlier. The ability of pyridoxamine-5-phosphate to activate apo-GOT is shown in figures 9 and 10. Under these activation conditions, it was possible to reactivate apo-GOT to 100% of its original activity. The Lineweaver-Burke plot, shown in figure 9, was used to determine the K_a of apo-GOT for pyridoxamine phosphate. Under the activation conditions described above, a K_a value of approximately 20 μM was obtained for pyridoxamine phosphate. A K_a value of 1.0 μM has been reported by O'Kane and Gunsalus (33).

Removal of Ammonia and AMP from Threonine Dehydrase

Ammonia and AMP were removed from threonine dehydrase by column chromatography with Sephadex G-25 (fine) according to the method of Rabinowitz (34). The column (20 x 0.7 cm.) was poured and equilibrated with potassium phosphate buffer (0.1 M, pH 8.0) containing 1 mM dithiothreitol. The enzyme sample (0.25 ml.) was passed through the column with the same buffer. Figure 11 shows a typical elution profile for this column.

Purification of Threonine Dehydrase

A mutant strain of *Escherichia coli* (LA-9) deficient in biosynthetic L-threonine dehydrase, was grown as described in the methods section. Biodegradative L-threonine dehydrase was purified from this strain of *E. coli* according to an unpublished method of Le Blond and Wood (35).

Figure 9. Activation of apo-glutamate-oxalacetate transaminase by pyridoxamine-5-phosphate (●—●). Also included is a Lineweaver-Burke plot (○—○) of the same data.

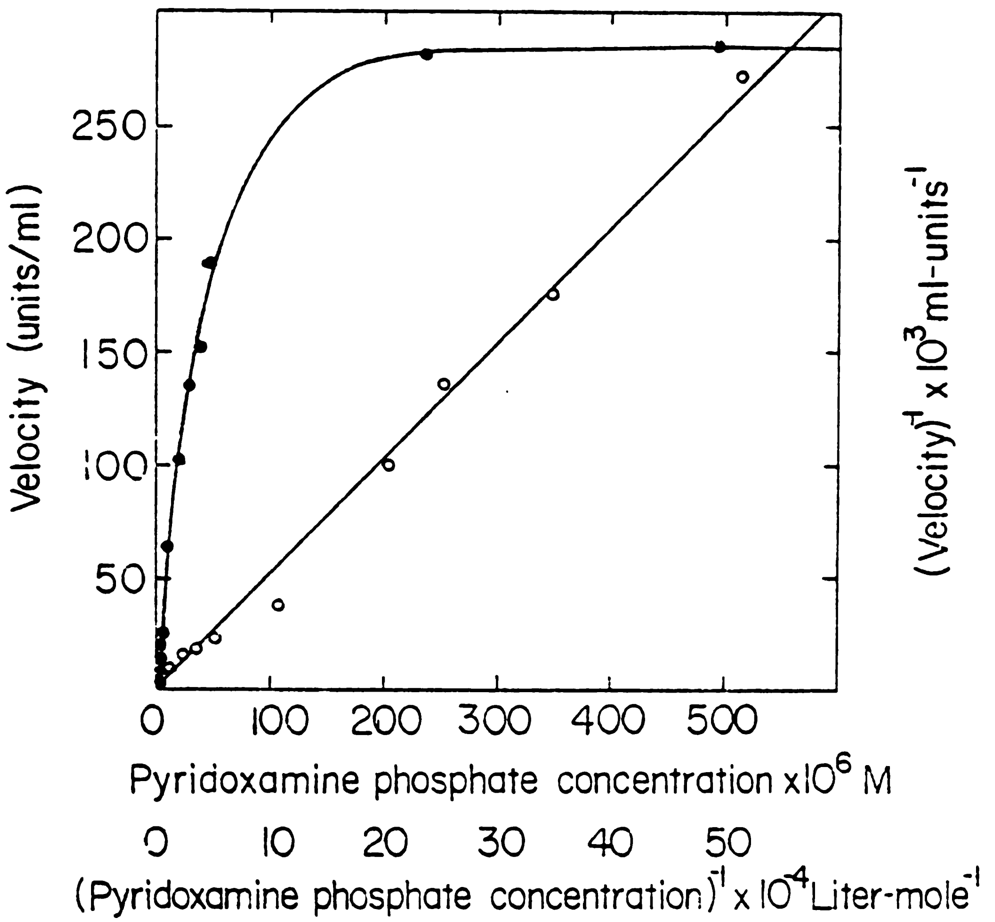

250
200
150
100
50
Velocity (units/ml)
(Velocity)$^{-1}$ x 10^{3} ml-units^{-1}
0
100
200
300
400
500
Pyridoxamine phosphate concentration x10^{6} M
0
10
20
30
40
50
(Pyridoxamine phosphate concentration)$^{-1}$ x 10^{-4} Liter-mole^{-1}

Figure 9

Figure 10. Standard curve for the activation of apo-glutamate-oxalacetate transaminase with pyridoxamine-5-phosphate.

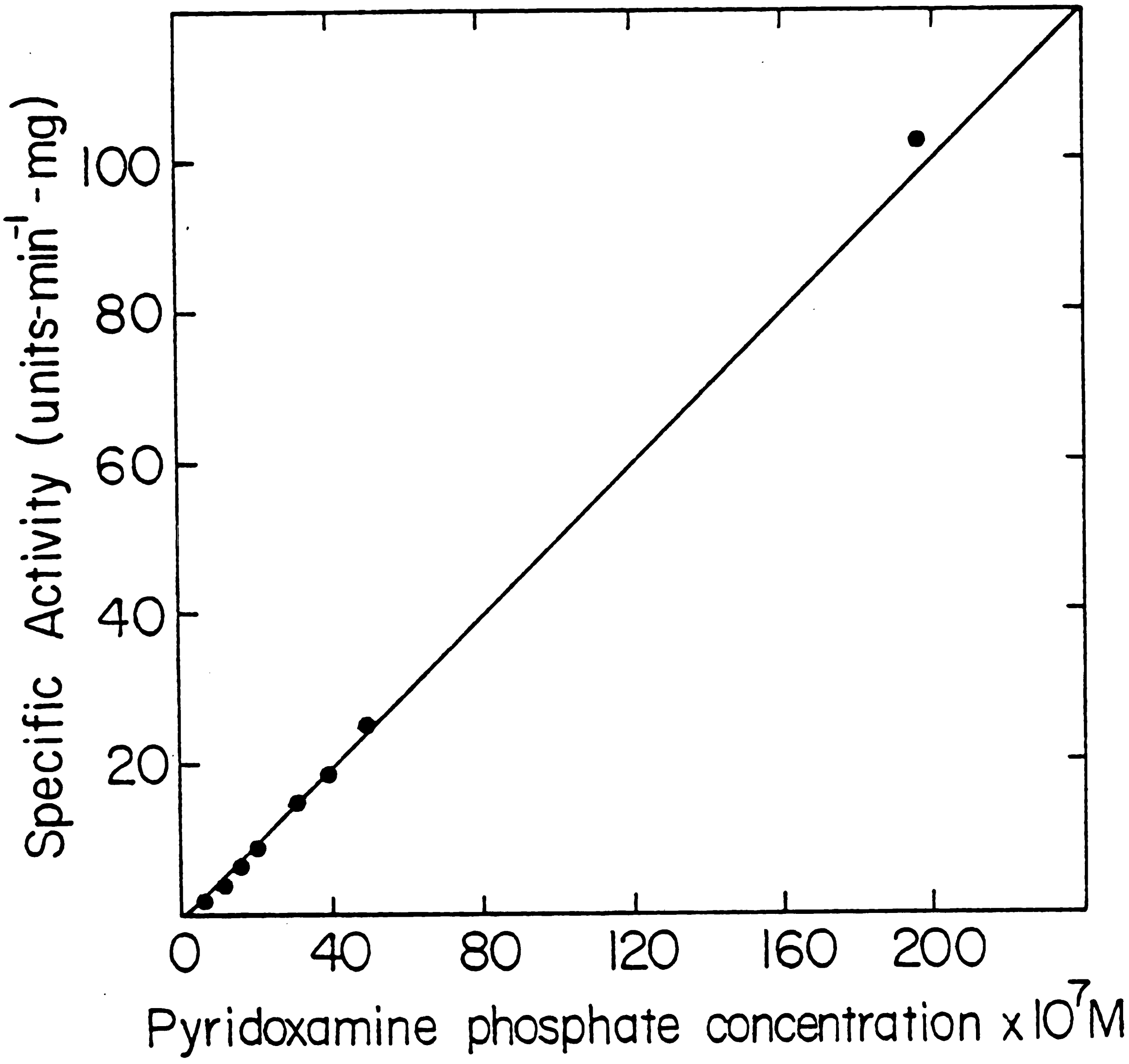

Specific Activity (units-min^{-1}-mg)
100
80
60
40
20
0
40
80
120
160
200
Pyridoxamine phosphate concentration x 10^{7}M

Figure 10

Figure 11. This figure shows the elution profile for the Sephadex G-25 column which was used to remove ammonia and AMP (○—○) from threonine dehydrase (●—●).

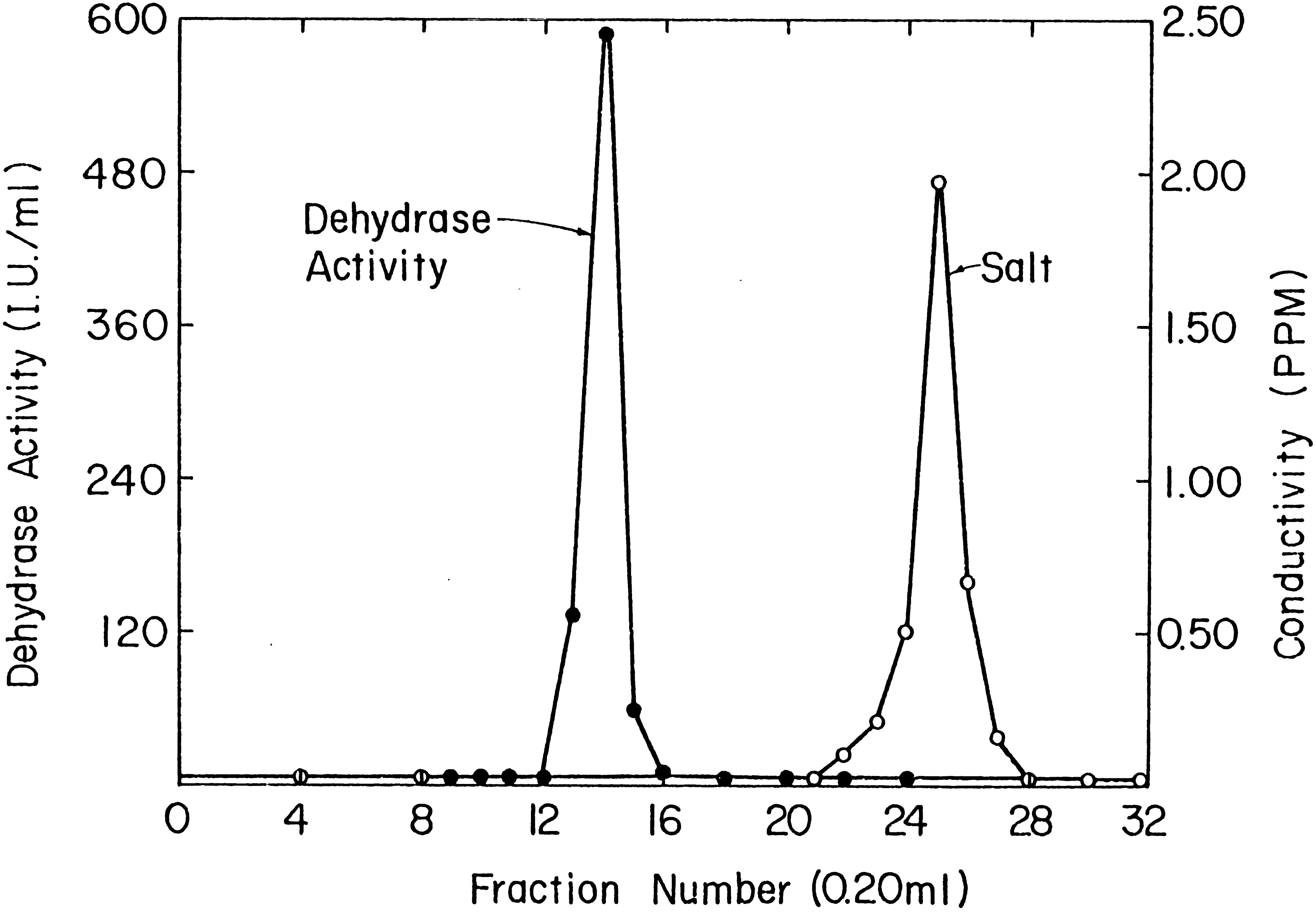

Dehydrase Activity (I.U./ml)
600
480
360
240
120
Conductivity (PPM)
2.50
2.00
1.50
1.00
0.50
Dehydrase Activity
Salt
0
4
8
12
16
20
24
28
32
Fraction Number (0.20ml)

Figure 11

Crude Extract

Frozen *E. coli* cells were suspended in sonication buffer [containing 1.0 mM ATP, 0.1 mM ADP, 0.1 mM NADP$^+$, 0.2 mM NAD$^+$, and 0.4 ml. phenylmethylsulfonyl fluoride (25 mg/ml. in butanol)], on the basis of 1.0 ml. of buffer per 1.0 g of cells. The cells were maintained at a temperature below 5°C. at all times during the fractionation process. The cells were disrupted by 15 3-minute pulses at 80 to 85 watts using a Branson Sonifier-Cell Disrupter. Following sonication, the suspension was centrifuged at 20,000 rpm for 90 minutes to remove cell debris.

AMP-Sepharose Affinity Chromatography

The supernatant from the previous step was applied directly to an AMP-Sepharose column (9.0 x 2.5 cm.). The AMP ligand was attached through its N^6 nitrogen atom of the adenosine ring to a hexane arm of Sepharose-4B (P-L Laboratories, Sepharose-4B-hexane-[N^6]AMP, type 2). The dolumn was washed by the sequential application of potassium phosphate buffers (0.1 M, pH 6.8) containing 5 mM dithiothreitol, and the following additional compounds: (a) 1 mM ATP, 0.01 mM ADP, 0.1 mM NADP$^+$, and 0.2 mM NAD$^+$, 80 ml., (b) 0.4 M potassium chloride, 40 ml., and finally (c) potassium phosphate buffer containing 5 mM dithiothreitol. This final wash was continued until the absorbance at 280 nm became constant.

The enzyme was then eluted from the column by means of a 0 to 100 mM AMP continuous gradient. The gradient was prepared using potassium phosphate buffer (0.1 M, pH 6.8) containing 5 mM dithiothreitol. A typical elution profile is shown in figure 12.

The fractions which contained the greatest yellow color were pooled. The enzyme was precipitated by the addition of 2.15 times the volume of the pooled fraction of a-monium sulfate (3.75 M, pH 7.0) containing 5 mM dithiothreitol, 5 mM AMP, and 1 mM EDTA. The precipitate was collected by centrifugation at 20,000 rpm for 1 hour. The pellet was resuspended in approximately 3 ml. of potassium phosphate buffer (1.0 M, pH 8.0) containing 1 mM dithiothreitol, and 5 mM AMP.

Sephadex G-200 Column Chromatography

The enzyme solution from the previous step was applied to a Sephadex G-200 column (90 x 2.5 cm.) which had been equilibrated with the same buffer. The activity was eluted by ascending chromatography with the same buffer. Figure 13 shows the elution profile for this column.

The fractions from the G-200 column with the most intense yellow color were pooled. The enzyme was precipitated by the addition of ammonium sulfate (as above) and centrifuged at 20,000 rpm for 1 hour. The pellet was resuspended in a minimal volume of potassium phosphate buffer (0.1 M, pH 8.0) containing 1 mM dithiothreitol, and 5 mM AMP. The enzyme solution was stored at -20°C.

Figure 12. Elution profile for threonine dehydrase from an AMP-Sepharose column. Relative fluorescence (●—●) and threonine dehydrase activity (▲—▲).

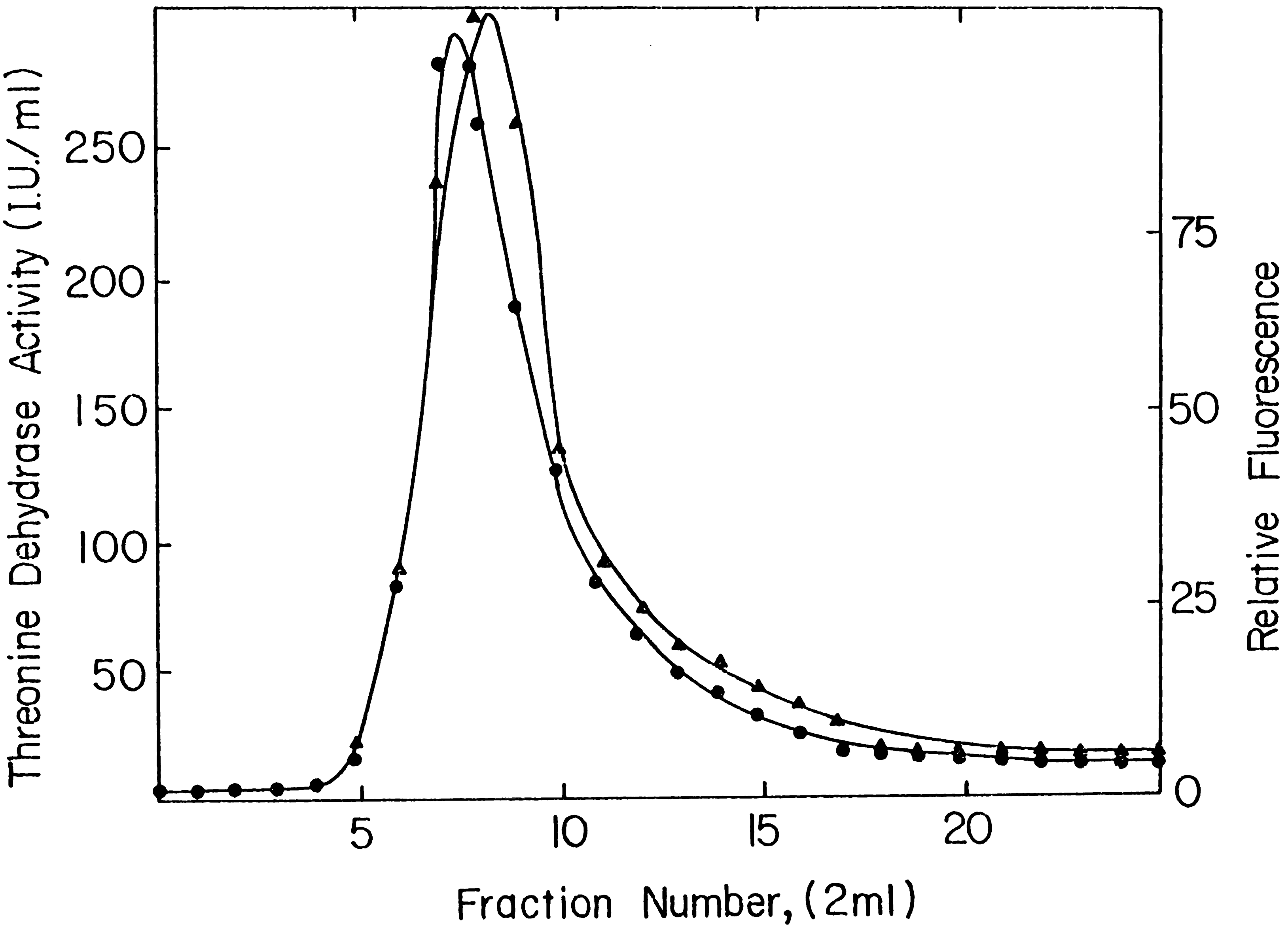

Threonine Dehydrase Activity (I.U./ml)
250
200
150
100
50
Relative Fluorescence
75
50
25
0
5
10
15
20
Fraction Number, (2ml)

Figure 12

Figure 13. Elution profile for threonine dehydrase from a Sephadex G-200 column. Relative fluorescence (●—●) and threonine dehydrase activity (▲—▲).

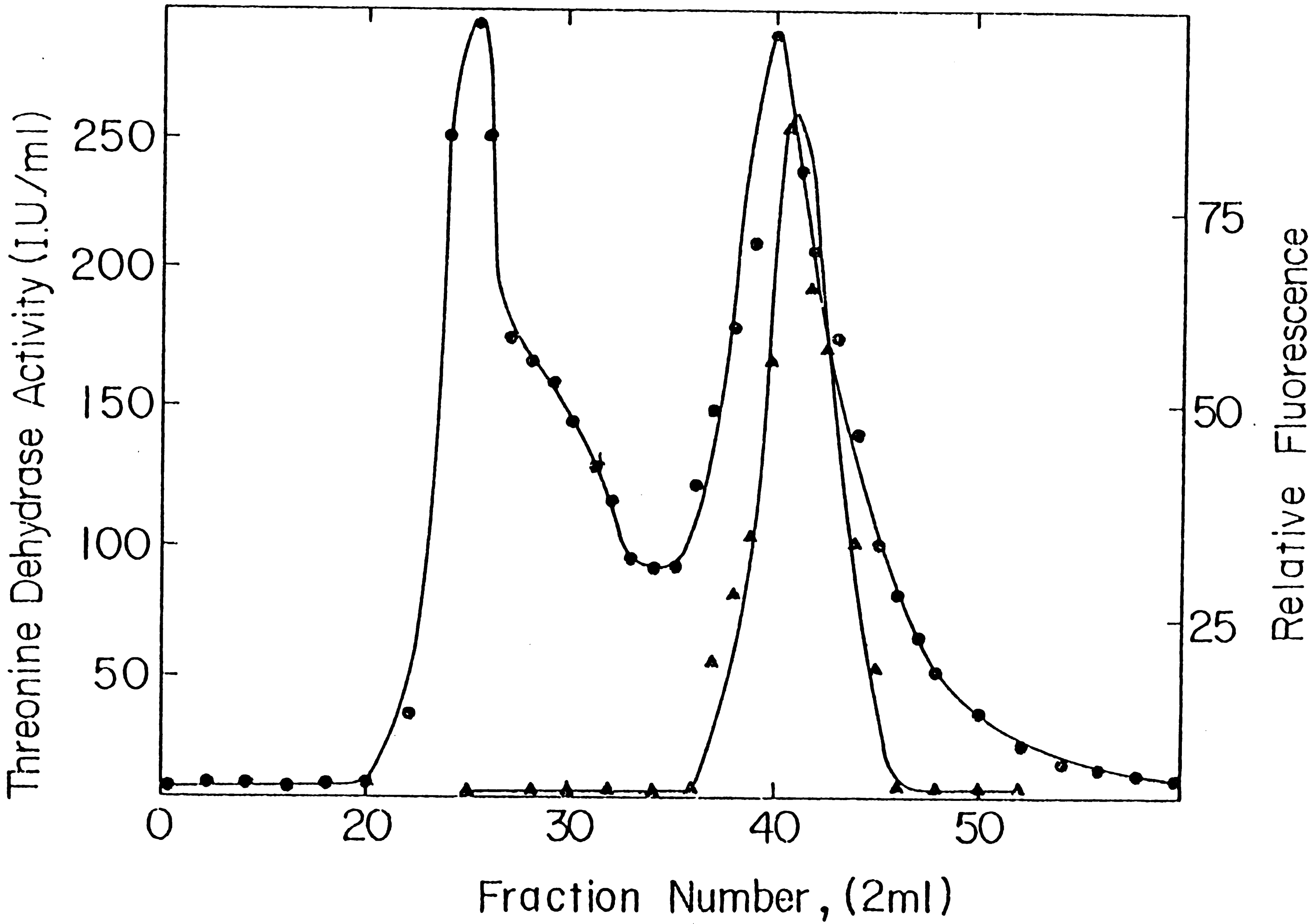

Threonine Dehydrase Activity (I.U/ml)
250
200
150
100
50
0
20
30
40
50
Fraction Number, (2ml)
Relative Fluorescence
75
50
25

Figure 13

RESULTS

It was the intent of this research project to try to establish whether or not an appreciable amount of pyridoximine phosphate was formed on threonine dehydrase in the presence of high levels of ammonia. The experimental approach used here was two-fold: (1) An attempt was made to trap, by sodium borohydride reduction, any pyridoximine phosphate which may be formed when threonine dehydrase was incubated with high levels of ammonia. The reduction product so formed would be pyridoxamine phosphate as a result of borohydride reduction of pyridoximine phosphate, as determined by its ability to activate resolved glutamate-oxalacetate transaminase; (2) Since the dehydrase has a characteristic absorption maximum near 410 nm., and near that of pyridoximine phosphate, an attempt was made to detect the formation of pyridoximine phosphate by difference spectroscopy.

Evidence for the Enzymatic Formation of Pyridoximine Phosphate

Free ammonia and AMP were removed from the threonine dehydrase by chromatography on Sephadex G-25, as described in the Methods section. This enzyme was then immediately used in the various experiments. In a typical experiment, threonine dehydrase (7.5 units) was incubated with 1.5 M ammonium chloride for 30 minutes at 4°C. Sodium borohydride was then added to a final concentration of 0.1 mg/ml, and the solution allowed to stand for an additional 30 minutes at 4°C. The final volume of the reaction mixture in all experiments was 0.1 ml. The solution was then heated to 90° to 100°C. for 15 minutes to destroy excess unreacted borohydride and to denature totally the threonine dehydrase. The denatured protein was removed by centrifugation and the supernatant was assayed for its ability to activate resolved glutamate-oxalacetate transaminase.

Two control experiments were also performed. In the first, threonine dehydrase was treated with sodium borohydride and incubated for 30 minutes at 4°C. In the second control, the incubation mixture was made 1.5 M in ammonium chloride, following sodium borohydride reduction, and allowed to stand for an additional 30 minutes at 4°C. Both controls were then treated in the same manner, as were the previous samples, to destroy excess borohydride and to denature the protein from the samples. All experiments were performed both in the presence and absence of AMP.

The results of these experiments are listed in Table 2. All experiments were performed in triplicate. The results presented here do indicate that when threonine dehydrase is incubated in the presence of ammonia, there is an appreciable amount of pyridoximine phosphate formed above that formed in the control.

Table 2. Formation of pyridoximine phosphate from threonine dehydrase and ammonia. (All tubes contained 75 mM potassium phosphate buffer, pH 8.0, 1 mM DTT, 7.5 units of threonine dehydrase, 0.1 mg/ml sodium borohydride, and 1.5 M ammonium chloride. The final volume in each tube was 0.1 ml. The concentration of AMP, when used, was 5 mM. The results are expressed as μM pyridoxamine phosphate ± the standard deviation.)

Table 2

First Addition	Incubation time (min)	Second Addition	Incubation time (min)	-AMP		+AMP	
				$PyrNH_2PO_4$ (μM)	Average ± SD	$PyrNH_2PO_4$ (μM)	Average ± SD
$NaBH_4$	30	---	30	1.05		1.44	
$NaBH_4$	30	---	30	0.88	0.85±0.20	0.95	1.36±0.37
$NaBH_4$	30	---	30	0.66		1.68	
$NaBH_4$	30	NH_4Cl	30	0.61		1.97	
$NaBH_4$	30	NH_4Cl	30	0.32	0.51±0.16	2.18	1.85±0.40
$NaBH_4$	30	NH_4Cl	30	0.59		1.41	
NH_4Cl	30	$NaBH_4$	30	4.24		5.33	
NH_4Cl	30	$NaBH_4$	30	6.37	5.24±1.07	6.61	5.51±1.02
NH_4Cl	30	$NaBH_4$	30	5.11		4.60	

Effect of Ammonia Upon the Absorption Spectrum of Threonine Dehydrase

Two approaches were used to evaluate the effect of ammonia upon the absorption spectrum of threonine dehydrase. First, the actual absorption spectrum (where the reference cuvette contained buffer only) was examined. The second approach involved an examination of the effect of ammonia upon the difference spectrum of threonine dehydrase (here both the reference and the sample cuvettes contained enzyme). The latter approach would allow for the resolution of an absorption peak, due to an interaction between threonine dehydrase and ammonia, which might occur at a wavelength very near that for the enzyme and thus be obscured in the absorption band for the enzyme.

Free ammonia and AMP were removed from threonine dehydrase by chromatography on Sephades G-25, as described in the Methods section. In a typical experiment, threonine dehydrase (1.25 mg/ml) was incubated with ammonium chloride (3 M) for 30 minutes at 4°C. In addition, two control experiments were performed. The first control, threonine dehydrase was reduced by preincubation with sodium borohydride (30 µg/ml) for 30 minutes at 4°C. before being incubated with ammonium chloride (3 M0. In the second control, threonine dehydrase was incubated in the presence of a-monium chloride (3 M) for 30 minutes at 4°C., and then sodium borohydride (30 µg/ml) was added and the solution incubated for an additional 30 minutes at 4°C.

Following the final incubation period, the various samples were transferred into cuvettes and the two types of spectra were recorded. All experiments were performed both in the presence and absence of AMP. For the two control experiments, sodium borohydride reduced-threonine dehydrase was used in the reference cuvette.

Absorption Spectra

The effect of ammonia upon the absorption spectrum of threonine dehydrase, both in the presence and absence of AMP, is shown in figure 14. The results here indicate that in the presence of high levels of ammonia, the absorption spectrum of threonine dehydrase becomes a very broad intense band between 350 and 440 nm. AMP has no effect upon the absorption spectrum. These spectra do not show any specific absorption phenomena in that there are no sharp, well defined peaks.

Difference Spectra

The conditions used here were very similar to those for determining the absorption spectra except that the absorption due to threonine dehydrase was concealed by placing enzyme in the reference as well as the sample cuvette. As a result of this, absorption maxima now occurring would be due to the interaction between ammonia and the enzyme only.

The difference spectra are shown in figures 15 and 16, for the presence and absence of AMP, respectively.

Figure 14. The effect of ammonia upon the absorption spectrum of threonine dehydrase. (Curve 1 represents the absorption spectrum of threonine dehydrase. Curve 2 represents the absorption spectrum of sodium borohydride-reduced threonine dehydrase. Curve 3 represents the absorption spectrum of threonine dehydrase in the presence of ammonium chloride. The sample cuvette contained potassium phosphate buffer, pH 8.0, 1 mM DTT, and 1.25 mg/ml threonine dehydrase. When used, the final concentration of AMP was 5 mM, sodium borohydride 0.03 mg/ml, and ammonium chloride 3 M. The contents of the reference cuvette were identical except that enzyme was omitted.

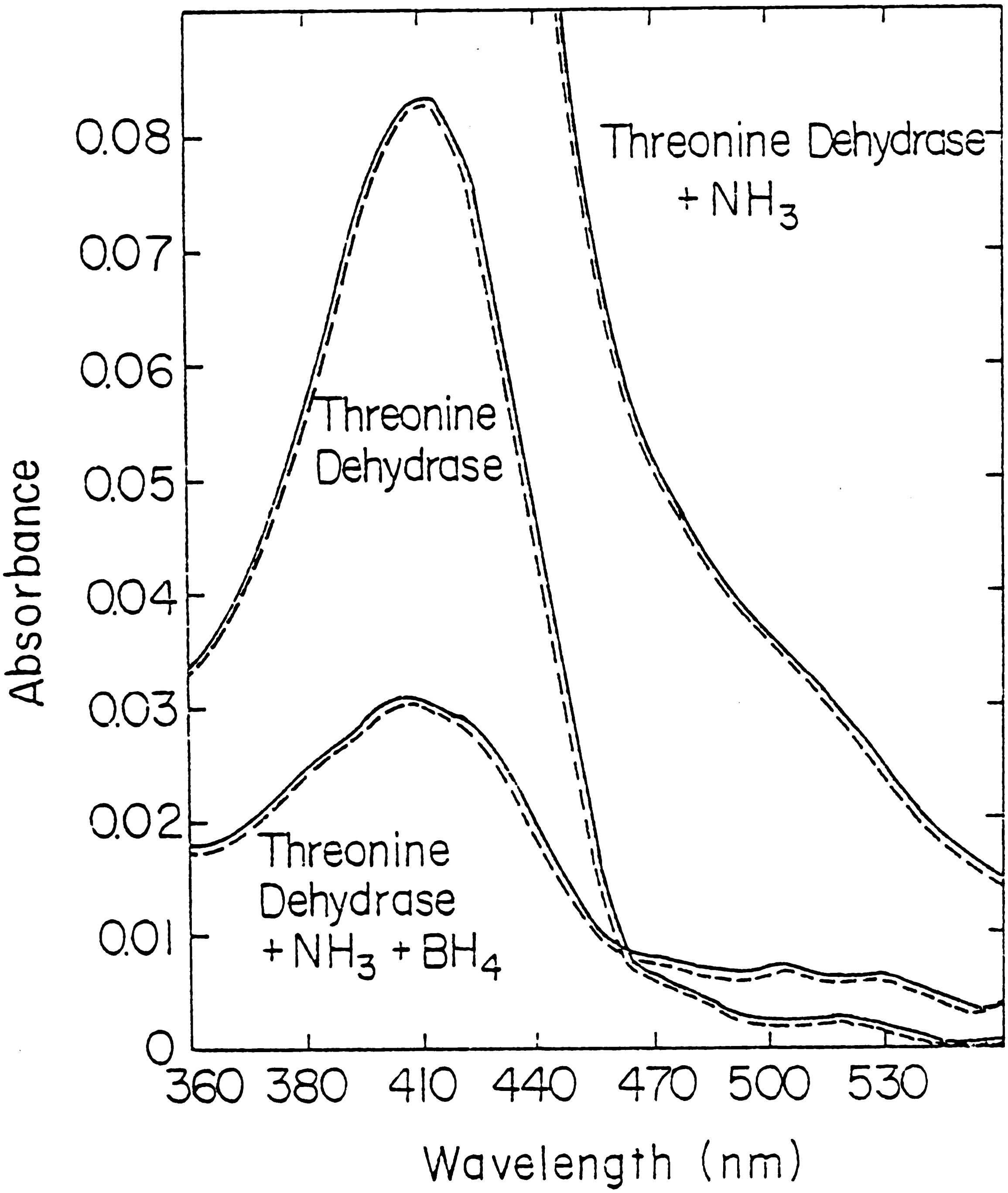

Absorbance
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
360
380
410
440
470
500
530
Wavelength (nm)
Threonine Dehydrase
+ NH$_3$
Threonine Dehydrase
Threonine Dehydrase
+ NH$_3$ + BH$_4$

Figure 14

Figure 15. The effect of ammonium ion upon the difference spectra of threonine dehydrase in the presence of AMP. (All experiments were performed in 75 mM potassium phosphate buffer, pH 8.0, containing 5 mM AMP and 1 mM DTT. The concentration of threonine dehydrase was 1.25 mg/ml. When used, the concentration of ammonium chloride was 3 M and sodium borohydride was 0.03 mg/ml. The final volume for all experiments was 0.3 ml. For all spectra shown here, compartment 2 of the reference cuvette contained phosphate buffer and ammonium chloride, while compartment 2 of the sample cuvette contained phosphate buffer only. (A) The effect of ammonia upon the difference spectrum of threonine dehydrase. Compartment 1 of the reference cuvette contained phosphate buffer and threonine dehydrase. The sample cuvette contained phosphate buffer, threonine dehydrase, and ammonium chloride. (B) The effect of ammonia upon the difference spectrum of sodium borohydride-reduced threonine dehydrase. Compartment 1 of the reference cuvette contained phosphate buffer, threonine dehydrase, and sodium borohydride. Compartment 1 of the sample cuvette contained phosphate buffer, threonine dehydrase, sodium borohydride, and ammonium chloride. In this spectrum, the threonine dehydrase was reduced with sodium borohydride prior to incubation with ammonium chloride. (C) The cuvettes contained the same solutions as in (B) except that the threonine dehydrase in the sample cuvette was incubated with ammonium chloride prior to reduction by sodium borohydride.) The dashed lines indicate the scanning of the same cuvettes 10 minutes after the first scanning.

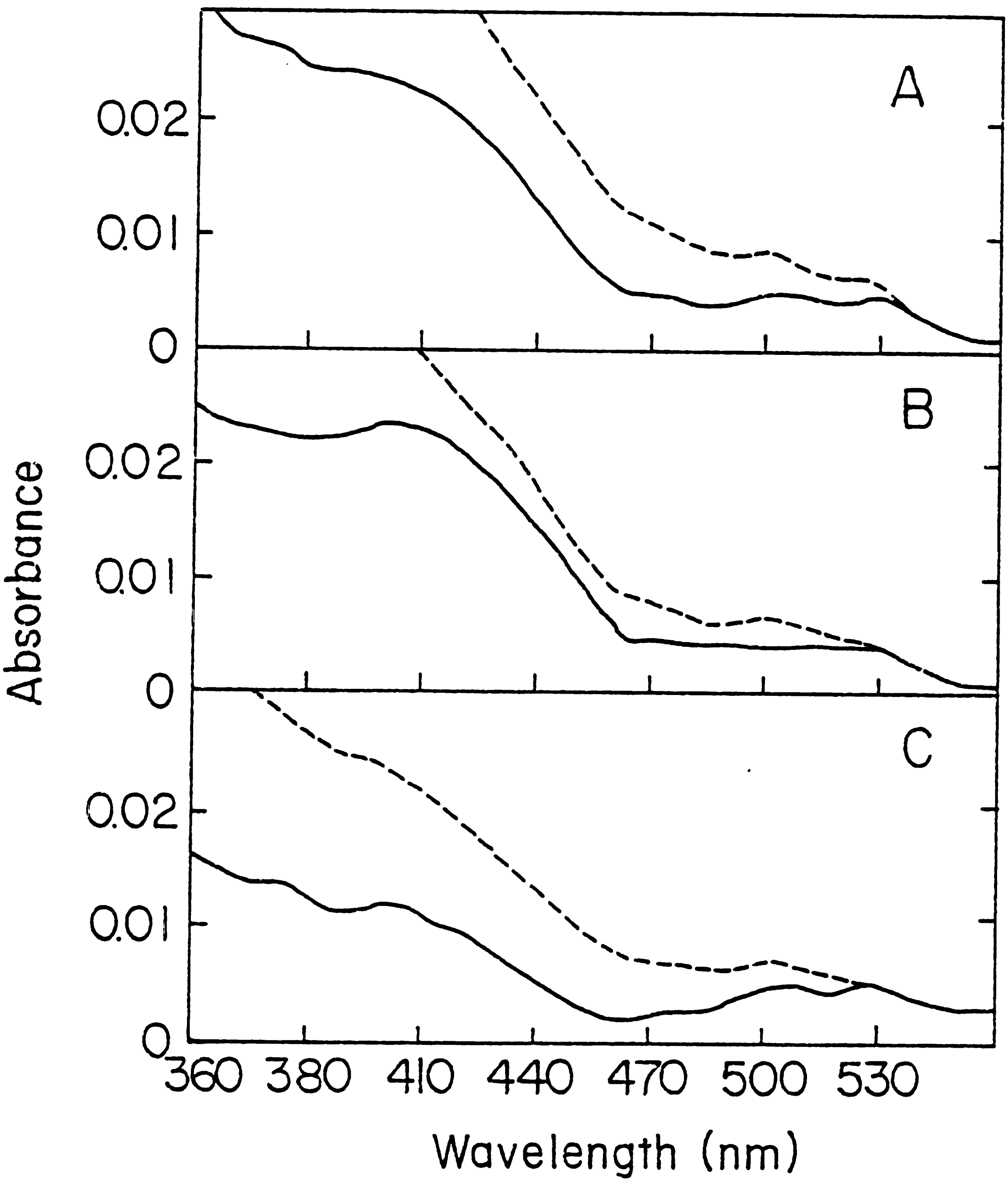

A
B
C
0.02
0.01
0
0.02
0.01
0
0.02
0.01
0
Absorbance
360
380
410
440
470
500
530
Wavelength (nm)

Figure 15

Figure 16. The effect of ammonium ion on the difference spectrum of threonine dehydrase in the absence of AMP. The experimental conditions are the same as described in figure 15 except AMP was omitted from all cuvettes.

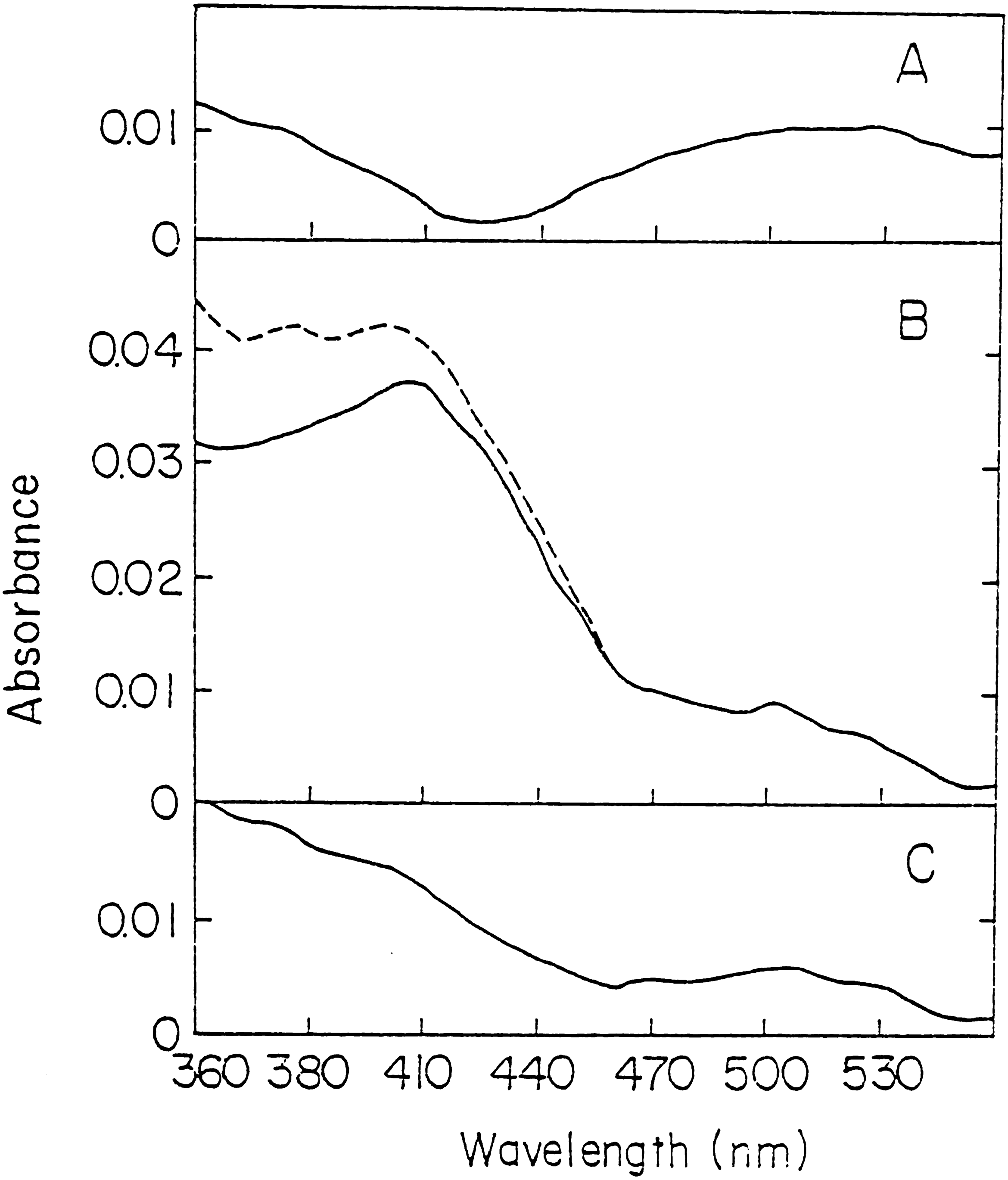

A
B
C
0.01
0
0.04
0.03
0.02
0.01
0
0.01
0
Absorbance
360
380
410
440
470
500
530
Wavelength (nm)

Figure 16

The difference spectra, like the absorption spectra, are difficult to interpret. The broadness of the absorption bands would again tend to indicate some type of nonspecific interaction between threonine dehydrase and ammonia.

DISCUSSION

The goal of this research was to determine if the incubation of threonine dehydrase in the presence of high levels of ammonia would lead to the formation of pyridoximine phosphate. The experimental approach used was twofold. First, an attempt was made to trap any pyridoximine phosphate which might be formed during the incubation by reduction with sodium borohydride, to pyridoxamine phosphate. Following denaturation and removal of the threonine dehydrase from the incubation mixture, this solution was assayed for its ability to activate resolved transaminase.

The second experimental approach involved determining if high levels of ammonia would effect the absorption spectra of threonine dehydrase. Difference spectra were also examined in an attempt to resolve any absorption, due to an interaction between ammonia and threonine dehydrase, which might occur at a wavelength near the absorption band characteristic of the enzyme and thus not be directly observable.

The results, shown in table 2, indicate that there is a significant amount of pyridoximine phosphate formed when threonine dehydrase is incubated with high levels of ammonia. When these results are expressed in terms of the fraction of total pyridoxal phosphate present, on a molar basis, which was converted to pyridoxamine phosphate, a value of approximately 17% is obtained. This value takes into a-count the fact that the threonine dehydrase used was about 60% pure.

The spectral experiments, which were performed, do not provide any additional evidence for the formation of pyridoximine phosphate during the incubation of threonine dehydrase with ammonia. The spectra, which were obtained, are not easily interpretable. The general appearance of the absorption bands are very broad and do not indicate any specific absorption maxima.

The result of this research indicates that enzyme-bound pyridoximine phosphate formation is not unique to the tryptophanase reaction mechanism. There is now a need to further evaluate the involvement of pyridoximine phosphate in the reaction mechanism for other pyridoxal phosphate-dependent enzymes. In particular, it is now of interest to determine if pyridoximine phosphate might be a common intermediate for all enzyme catalyzed α,β-elimination reactions. The following discussion describes some future experiments which should allow a more detailed evaluation of the role of pyridoximine phosphate in pyridoxal phosphate-mediated enzymatic catalysis.

For threonine dehydrase, it is now necessary to more accurately establish the amount of pyridoximine phosphate which is generated when the enzyme is incubated with ammonia. There is a need to establish the time course for the formation of this intermediate. More importantly, it is necessary to demonstrate directly, by purification, that the material which activates apo-transaminase is actually pyridoxamine phosphate. Finally, an attempt should be made to trap pyridoximine phosphate during the conversion of threonine to α-ketobutyrate by threonine dehydrase and also to determine if the presence of α-ketobutyrate decreases the accumulation of pyridoximine phosphate.

The same experimental approaches, which are described above for threonine dehydrase, should be used to determine if pyridoximine phosphate is formed in the reaction mechanism for other pyridoxal phosphate-dependent enzymes. These include tryptophanase, β-tyrosinase, D-serine dehydrase, various amino acid decarboxylases, and phosphorylase.

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