THE PURIFICATION AND PROPERTIES OF THE L-THREONINE DEHYDRASE OF SHEEP LIVER

by

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I. INTRODUCTION

Enzymes are proteins whose biological function is the catalysis of chemical reactions in living systems. Many of these enzymes contain non-protein components, coenzymes, which are essential for enzyme activity. One of these coenzymes, pyridoxal phosphate, is the active form of vitamin B_6. It is required for many types of biological reactions and is the key compound in some of the most important reactions of protein chemistry. One type of reaction utilizing pyridoxal phosphate is the elimination and replacement of substituents on the β-carbon of amino acids. The present study involves one of the many enzymes which carry out this type of reaction, the L-threonine dehydrase of sheep liver. By studying a specific reaction involving pyridoxal phosphate, it is hoped that our knowledge of the mechanism of action of pyridoxal phosphate enzymes will be increased.

The L-threonine dehydrase of sheep liver is an excellent enzyme for study because it is readily available and exceptionally stable. This enzyme converts L-threonine to α-ketobutyrate and ammonia, both of which are easily measured.

In order to carry out a detailed study of the enzyme, it is necessary to have it in a highly pure form. Therefore, the first part of this work concerns the purification

of the enzyme. Although a homogeneous preparation has not been obtained, the present purification procedure provides a convenient preparation of enzyme of high purity. In order to determine how much further work is necessary to obtain a homogeneous preparation, sedimentation and electrophoretic mobility determinations were performed.

A study was made of the stability and activity of the enzyme under various conditions of temperature and ionic environment. This information should be useful in any further attempts to purify the enzyme. In addition this study has provided some information concerning the relationship between the enzyme, coenzyme and their environment.

L-serine, a homolog of L-threonine, also acts as a substrate for the enzyme but inactivates the enzyme in the process. This inactivation is studied in some detail. The results of this study has led to the proposal of a modified mechanism involving the conversion of L-serine to pyruvate and ammonia.

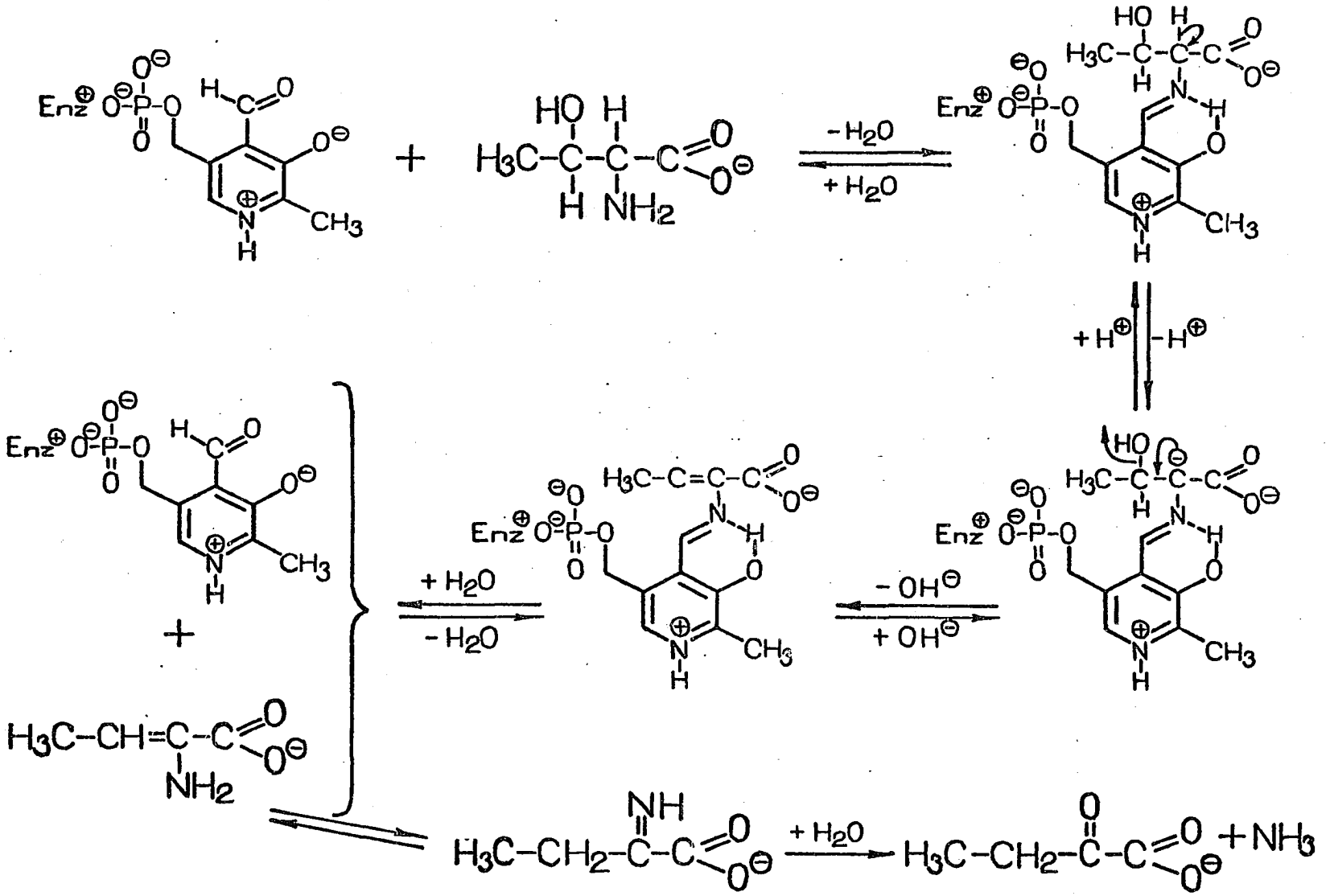
II. LITERATURE SURVEY

The first detailed study of the deamination of a β-hydroxy amino acid by bacterial cells and tissues was by Gale and Stephenson in 1938 (1). They followed the deamination of DL-serine by the release of ammonia in the presence of cells of Escherichia coli. This reaction proceeded anaerobically, thus distinquishing it from oxidative deamination. In 1943, Chargaff and Sprinson, showed that the β-hydroxyl group of serine was required by the enzyme for activity; replacement of the hydroxylic hydrogen by larger groups resulted in a loss of activity (2). Pyruvic acid was also isolated as a product of the deamination of serine. Therefore, a mechanism was postulated in which serine was dehydrated to α-aminoacrylic acid (2,3). This intermediate was tautomerized to the unstable imine, which on hydrolysis yielded pyruvate and ammonia. Although this basic mechanism is still accepted, it has been modified to include the role of pyridoxal phosphate. Dehydrases require pyridoxal phosphate as a coenzyme. Additional evidence for this basic mechanism has been presented by Phillips and Wood (4). In a deuterium oxide medium the L-threonine dehydrase of E. coli was shown to incorporate deuterium into the α-position of threonine as well as into the β-position of α-ketobutyrate. In the presence of borotritide,

α-aminobutyrate was formed with tritium exclusively on the α-carbon. All of the steps were reversible with the exception of the hydrolysis to products.

The accepted mechanism of the deamination of L-threonine, including the function of pyridoxal phosphate in this mechanism is shown in Fig. 1. No attempt is made to show the manner in which the coenzyme is bound to the apoenzyme. Although the exact mode of binding is not known, some insight into the probable binding of the coenzyme has been revealed for other pyridoxal phosphate enzymes. The principle place of binding appears to be through the carbonyl group of the coenzyme. In all of the cases studied, phosphorylase, homoserine dehydratase (also known as cystathionase), aspartate aminotransferase (also known as glutamic-oxaloacetic transaminase or glutamic-aspartic transaminase), serine transhydroxymethylase, and L-aspartate-4-decarboxylase, the carbonyl group of the coenzyme is not free (5-9). Instead it appears to be bound to the enzyme either as an aldimine or in a form that readily converts to such an aldimine. The aldimine structure is supported by the appearance of spectral peaks at one pH or another which have been attributed to a Schiff base linkage between the coenzyme and protein. Aspartate aminotransferase, phosphorylase, homoserine dehydratase, glutamic acid decarboxylase, and serine transhydroxymethylase reveal a

Fig. 1. The mechanism of the conversion of L-threonine to α-detobutyrate and ammonia by L-threonine dehydrase.


Enz
CH3
- H2O
+ H2O
+ H⊕
- H⊕
+ H2O
- H2O
- OH⊖
+ OH⊖
+ H2O
+ NH3

peak at 415 mμ to 430 mμ, which is superimposed over the spectrum of the protein (10-15). This peak shifts to 360 mμ in the case of aspartate aminotransferase, homoserine dehydratase, and glutamic acid decarboxylase, as the pH is increased (10,16,17). Reduction of the aldimine results in the loss of the 415 mμ to 430 mμ peak and, in addition, the loss of enzyme activity. The reduced Schiff base of phosphorylase, homoserine dehydratase, aspartate aminotransferase, serine transhydroxymethylase and L-aspartate-4-decarboxylase can be isolated following partial hydrolysis of the enzyme and the former carbonyl carbon is now covalently linked to the ε-amino group of lysine. Therefore, if the azomethine link between a lysine residue of the apoenzyme and pyridoxal phosphate pre-exists in the enzyme, the formation of the enzyme-substrate complex probably takes place by a process of "transaldimination" rather than by elimination of water (18,19). The phosphate group of the coenzyme also has been implicated as a site of binding resulting from its effectiveness in resolving aspartate aminotransferase and its inhibition of the recombination of apoenzyme and coenzyme (20,21). In addition, the affinity of the apoenzyme of aspartate aminotransferase for pyridoxal is about 1/10 of the affinity of the apoenzyme for pyridoxal phosphate (19). A correlation of the inhibitory potencies of various coenzyme analogs for different pyridoxal

phosphate enzymes reveals that every substituent grouping of pyridoxal phosphate affects the firmness of the binding of coenzyme and enzyme (22).

Since the enzymatic reaction results only in the removal of water, this type of enzyme has been called a dehydrase, which is the term used here. Other workers have used "deaminase" or "dehydratase" to refer to the same enzyme. The official nomenclature for this enzyme according to the International Union of Biochemistry and the International Union of Pure and Applied Chemistry is E.C. 4.2.1.16: L-threonine hydro-lyase (deaminating) (23).

In 1943, Chargaff and Sprinson showed that DL-threonine was converted to α-ketobutyrate and ammonia (3). Since then, dehydrases have been isolated which are specific for the individual isomers of serine and threonine. Escherichia coli is particularly interesting in this respect. The work of Wood and Gunsalus showed that the dehydrase of E. coli was most active towards L-threonine (24). Then Metzler and Snell readily separated a D-serine dehydrase from the L-serine or L-threonine dehydrase also present (25). Later, Umbarger and Brown isolated two distinct L-threonine dehydrases, one adaptive and the other constitutive (26). It is interesting to note that although dehydrases have been isolated with very different substrate requirements, no dehydrase has been isolated specific for

only one of the two substrates, threonine and serine.

All of the dehydrases studied thus far require pyridoxal phosphate as a coenzyme (25-32). This requirement is shown in the following ways. Many dehydrases require the coenzyme for maximum activity (25,27,33). In addition, the coenzyme can be resolved from the threonine dehydrases of Neurospora, the rumen microorganism LCI and sheep liver (27,29,32). The apoenzyme is catalytically inactive; activity is restored partially or totally, by incubation with added pyridoxal phosphate.

Additional methods have been used to show the requirement for pyridoxal phosphate in other vitamin B_6 enzymes. The use of spectral observations were discussed previously. It is also possible to release the coenzyme from the enzyme by treatment with concentrated acid or alkali and to isolate the coenzyme. This has been accomplished in the case of aspartate aminotransferase, phosphorylase, homoserine dehydratase, and serine transhydroxymethylase (10,15-17,34). The presence of pyridoxal phosphate bound to the enzyme also can be detected by microbiological assays (35).

Although pyridoxal phosphate appears to be the coenzyme associated with serine and threonine dehydrases, some of these enzymes require additional additives for maximum activity. Adenylic acid and glutathione activate the L-threonine dehydrase of E. coli (24,26). Glutathione also

activates the L-threonine dehydrase of the rumen microorganism LCI (29). Adenosine diphosphate activates the threonine dehydrase of Clostridium tetanomorphum (36). Monovalent cations, especially potassium and ammonium, activate many dehydrases (1,30,32,37-41). Since these enzymes also require pyridoxal phosphate, the other cofactors may play a structural role. This is apparently true in the case of the L-threonine dehydrase of E. coli, for which Phillips and Wood showed that the presence of adenylic acid favors the formation of a more active dimer (4,42).

Meister et al. studied L-aspartate-4-decarboxylase and aspartate aminotransferase and found that they are activated by α-keto acids, as well as by pyridoxal phosphate (35,43-47). The effects of the activation by pyridoxal phosphate and by α-keto acids are not additive. Both activators reverse the inhibition by amino acids. Meister postulated that the incubation of a vitamin B_6 enzyme with its substrate or substrate analog inactivates it as a result of the conversion of enzyme-pyridoxal phosphate to enzyme-pyridoxamine phosphate. This conversion would be reversed by α-keto acids or pyridoxal phosphate. Enzyme-pyridoxamine phosphate dissociates readily yielding apoenzyme, which would be reactivated by pyridoxal phosphate. Vitamin B_6 analogs, which could not form Schiff bases with substrates,

inhibit the reaction by replacing enzyme-pyridoxal phosphate but protect the enzyme from inactivation by retarding dissociation of pyridoxamine phosphate. Thus the enzyme catalyzes two reactions; one transamination, which can destroy or regenerate the coenzyme needed for the other reaction, in this case decarboxylation. Meister has suggested that all vitamin B_6 enzymes may have this ability to undergo transamination and that this ability may serve as a control mechanism.

The L-threonine dehydrase of sheep liver was first purified by Sayre and Greenberg in 1955 (48). Additional purification was accomplished by Nishimura and Greenberg and by Davis and Metzler (32,40,41). Both used similiar purification procedures consisting of three heat treatments, two ammonium sulfate fractionations and chromatography on diethylaminoethyl-cellulose. Nishimura also included an acetone precipitation step.

The sheep liver L-threonine dehydrase has been observed to have activity towards L-threonine, L-serine and L-allothreonine (32,40,41,49). Activity on L-allothreonine is apparently 0.23 times as rapid as that on L-threonine. As with the L-threonine dehydrase of E. coli, L-serine inactivates the enzyme within 5 minutes at pH 7 (24). At higher pH values, the enzyme is not completely inactivated after 5 minutes; product is still produced

but at a lower rate. Therefore, the enzyme is modified by the action of serine.

Other general properties of this enzyme include its resistance to heat; its activation by monovalent cations; its inhibition by carbonyl reagents; and its stability over a pH range of 5.5 to 9 (32,40,41,49). The enzyme is stable at 75°C for 15 minutes with no loss of activity. Monovalent metal ions, K^+, NH_4^+, Rb^+, Li^+, and Na^+, activate the enzyme in decreasing order. Carbonyl specific compounds, namely hydroxylamine and semicarbazide, competitively inhibit the enzyme. The Michaelis constant is pH dependent, whereas the maximum velocity is not. K_M has been calculated to be 8×10^{-4} M for L-threonine and approximately 4×10^{-3} M for L-serine at pH 9 (40,41).

Reviews on serine, threonine and homoserine dehydrases by Greenberg can be found in The Enzymes and in Methods of Enzymology (50,51). A recent review on pyridoxal phosphate, including both enzymatic and non-enzymatic studies, is also available (52).

In the purification of the L-threonine dehydrase of sheep liver advantage was taken of the relatively recent innovation of gel filtration on Sephadex. Sephadex consists of a three dimensional network of a crosslinked polysaccharide, dextran. This insoluble network has a nonionic character; however, it has some polar properties because

of the high content of hydroxyl groups (53). Its hydrophilic character causes it to swell considerably when mixed with an aqueous solution. The swelling is dependent on the degree of crosslinkage. A high degree of crosslinkage gives a compact structure with low porosity, whereas a low degree gives a highly porous structure.

The separation of substances on Sephadex is mainly caused by its molecular sieving properties, by which the smaller particles are retained by their diffusion into the gel grains and the larger particles pass through the column, since they are too large to diffuse through the gel. Once the smaller molecules have been washed through the column, no regeneration is necessary and the column can be used again without repacking.

The use of Sephadex was first described by Porath and Flodin (54). They showed its application to the desalting of proteins and the fractionation of a mixture of saccharides. Since then, over 200 papers have been published describing the use of Sephadex in various techniques and applications. These applications include the desalting of proteins (54-57); the fractionation of polymers according to their molecular weight (58-60); the separation of mixtures by zone precipitation (61); the concentration of solutes of high molecular weight (62,63); and its use as a support for thin layer chromatography and electrophoresis

(64-67).

Ionic groups have also been introduced into Sephadex, coupling ion exchange properties with molecular sieving properties. Three ion exchange Sephadex gels are available. They include an anionic gel, containing the diethylaminoethyl (DEAE) radical and two cationic gels, containing the carboxymethyl and sulfonylethyl radicals.

In 1961, Porath separated the two basic polypeptides oxytocin and vasopressin by chromatography on DEAE-Sephadex (68). Since then the ion exchange Sephadex gels have been used extensively in the purification of proteins. Quite frequently multiple fractions of enzymes have been obtained (69-72). DEAE-Sephadex has also been used for thin layer chromatography (73).

Additional literature, including a series of reference cards containing abstracts of publications on the use of Sephadex and Sephadex ion exchangers, can be obtained from Pharmacia Fine Chemicals, New Market, N.J.

AG11A8 is a new ion retardation resin for the chromatographic desalting of biochemicals. This resin is made by polymerizing acrylic acid inside Dowex 1 to form polymer beads containing paired anion and cation sites. These sites attract mobile anions and cations and associate weakly with them. As a result, AG11A8 absorbs both anions and cations in equivalent amounts, leaving a neutral

environment in contrast to the acidic and basic environments of conventional ion exchange resins. The absorbed salts can be eluted with water and the column used again.

The use of polyacrylamide gels for disc gel electrophoresis is another application of the property of molecular sieving. Polyacrylamide gels are thermostable, transparent, strong, relatively inert, nonionic and can be prepared with a wide range of pore sizes. The viscosity of the gel is dependent on its pore size relative to the radius of the particle. This introduces a molecular sieving effect and enables particles with the same mobility but of different size to be separated. This technique takes advantage of the effects of discontinutities in pH and gel pore size. The proteins first enter a large pore gel in which they are sorted out according to their mobilities into highly concentrated discs. These discs pass into a small pore gel where further separation takes place.

The theory of disc gel electrophoresis has been described by Ornstein (74). Disc gel electrophoresis has been used as an analytical tool for both clinical work and research. Normal human serum is separated into about 25 discrete bands (75,76). Crystalline protein preparations, e.g. trypsin, chymotrypsin, ribonuclease, protamine sulfate, globin and lysine vasopressin have been shown to contain more than one component (77). Disc gel electrophoresis has

been compared to starch gel and agar electrophoresis (78, 79). The results indicate that disc gel electrophoresis gives similiar separations, easier, faster, and with less cost per experiment.

III. EXPERIMENTAL

A. Materials and Equipment

1. Materials

a. Buffered substrate, 0.1 M, pH 8.9. A mixture of 0.2978 gm. L-threonine or 0.2627 gm. L-serine and 1.05 meq. of sodium hydroxide was prepared and diluted to 25 ml.

b. Buffered substrate, 0.1 M, pH 7.2. A solution of 0.2978 gm. L-threonine or 0.2627 gm. L-serine in 25 ml. of 0.1 M phosphate buffer, pH 7.2 was prepared.

c. Tris-carbonate buffer, 0.5 M, pH 8.95. A mixture of 6.05 gm. recrystallized tris(hydroxymethyl)aminomethane, 4.2 gm. sodium bicarbonate and 2.3 meq. of hydrochloric acid was prepared and diluted to 100 ml.

d. Potassium phosphate buffer, 0.5 M, pH 7.2. A mixture of 61.27 gm. potassium monobasic phosphate and 19.33 gm. potassium dibasic phosphate was prepared and diluted to one liter. This solution was diluted to prepare phosphate buffers of lesser concentration.

e. Trichloroacetic acid, 25%. A solution of 25 gm. trichloroacetic acid in 100 ml. of distilled water was prepared.

f. 2,4-Dinitrophenylhydrazine reagent. A solution of 0.250 gm. recrystallized 2,4-dinitrophenylhydrazine in

500 ml. of 2.0 N hydrochloric acid was prepared and filtered.

g. Sodium hydroxide, 2.5 N. A solution of 100 gm. reagent grade sodium hydroxide in one liter of boiled, deionized, distilled water was prepared.

h. Nesslers solution, No. 1. A mixture of 35 gm. potassium iodide, 50 gm. mercuric iodide and 300 meq. of sodium hydroxide was prepared and diluted to one liter.

i. Nesslers solution, No. 2. A mixture of 4 gm. potassium iodide, 4 gm. mercuric iodide and 1.75 gm. gum ghatti was prepared and diluted to one liter with ammonia free distilled water.

j. Molybdenum reagent. A mixture of 5 gm. ammonium molybdate and 0.15 meq. of sulfuric acid was prepared and diluted to 100 ml.

k. Ninhydrin solution. A solution of 0.80 gm. stannous chloride in 50 ml. of 0.20 M sodium citrate buffer, pH 5.0 was prepared. This solution was added to 2 gm. ninhydrin in 50 ml. of methyl cellosolve.

l. Saturated ammonium sulfate, pH 7.3. Ammonium sulfate was added to boiling distilled water until no more would dissolve. The cooled supernatant was decanted and a diluted sample (1/5) was adjusted to pH 7.3 using concentrated ammonia. The saturated supernatant was likewise adjusted based on the diluted sample.

m. Pyridoxal phosphate, 0.001 M. A solution of

0.0067 gm. pyridoxal phosphate, monohydrate in 25 ml. of 0.1 M phosphate buffer, pH 7.2 was prepared and stored in a freezer.

n. Sheep liver. Fresh sheep liver was brought from Iowa Packing Co.; Des Moines, Iowa, packed in ice and immediately used or frozen for future use. In later experiments, fresh sheep liver was shipped in dry ice from Wilson Packing Co.; Omaha, Nebraska and remained frozen until used. Arrangements were always made to secure fresh, mature sheep livers. L-threonine dehydrase was also prepared in the laboratory of Dr. Leodis Davis; Howard University; Washington, D.C.

o. Sephadex and Diethylaminoethyl-Sephadex. Sephadex and DEAE-Sephadex were obtained from Pharmacia Fine Chemicals; New Market, N.J.

p. Aquacide. Aquacide I and II were obtained from Calbiochem; Los Angeles, Calif.

q. AG11A8, ion retardation resin. AG11A8, ion retardation resin was obtained from Bio Rad Laboratories; Richmond, Calif.

2. Apparatus

a. Disc gel electrophoresis. Equipment and solutions were obtained from Canal Industrial Corporation; Bethesda, Md.

b. Collodion tube and apparatus. Collodion tubes

and apparatus were obtained from Carl Schleicher and Schuell Co.; Keene, N.H.

B. Analytical Methods

1. Enzyme Assay

A mixture of 0.9 ml. of enzyme solution and 0.3 ml. of 0.5 M tris-carbonate buffer, pH 8.95, was placed in a constant temperature bath, at 37°C. The buffered substrate was also placed in the bath. After 5 minutes, 0.3 ml. of 0.1 M buffered substrate, pH 8.9 was added to the mixture and incubated for 30 minutes. The reaction was stopped by the addition of 0.5 ml. of 25% trichloroacetic acid. After centrifugation, one ml. of the supernatant was assayed for keto acid by the method of Sayre and Greenberg (48). Two ml. of 2,4-dinitrophenylhydrazine reagent were added to the ml. of supernatant. After 15 minutes, 2 ml. of 95% ethanol and 5 ml. of 2.5 N sodium hydroxide were added. The solution was allowed to stand for 10 minutes to develop the color and the optical density was read at 520 mμ in a Beckman Model DU spectrophotometer against a denatured enzyme blank. The α-keto acid present was determined from a standard curve. An optical density of 1.00 was equivalent to 1.20 micromoles of α-ketobutyrate or 1.00 micromole of pyruvate. The production of α-keto acid was linear with time. Therefore, the number of enzyme units

were computed by multiplying the absorbancy at 520 mμ by the factors 1.11 X 2 X 2 X 1.20 corresponding to the aliquot size, the reaction time and the conversion factor obtained from the standard curve. When L-serine was used as the buffered substrate, the reaction time was one minute; therefore the factor 2 was replaced by 60 and the conversion factor 1.20 was replaced by 1.00.

2. Protein determination

During the purification the absorbancy of a diluted enzyme solution at 280 mμ was measured as an indication of the content of protein and other light absorbing material. As the purification of the enzyme increased, an optical density of 1.00 approached an equivalent of 1 mgm. protein (32).

3. Unit of enzyme

A unit of enzyme was arbitrarily defined as that amount of enzyme producing one micromole of α-keto acid in one hour at 37°C, pH 8.9. The unit of enzyme as defined by the International Union of Biochemistry is that amount of enzyme which will catalyse the transformation of one micromole of substrate per minute under optimum conditions of pH and substrate concentration (23). Therefore the unit used here is 1/60 of the International unit.

4. Purity index

The purity index was defined as the units of enzyme

per ml. divided by the optical density of the enzyme solution at 280 mμ.

5. Methods of concentrating the enzyme

a. 60% Saturated ammonium sulfate precipitation, pH 7.2. The enzyme solution was made 60% saturated with ammonium sulfate at 25°C by adding 390 gm. salt per liter of solution. Then the pH was adjusted to 7.2 using concentrated ammonia. The solution was allowed to stand at least five hours and the precipitate was dissolved in the desired volume of 0.1 M phosphate buffer, pH 7.2.

b. Dialysis against saturated ammonium sulfate solution, pH 7.3. The enzyme was dialyzed against a saturated ammonium sulfate solution pH 7.3 for at least 3 hours. The precipitate was dissolved in the desired volume of 0.1 M phosphate buffer, pH 7.2.

c. Vacuum filtration using collodion tubes. The apparatus consisted of a large glass tube closed at one end with a side arm near the open end; a small glass tube opened at both ends of which one end was tapered; a rubber stopper with a hole in it containing an adapter for the tapered end of the small glass tube; and a collodion tube capable of retaining particles larger than 5 millimicrons.

The large glass tube was filled with buffer to a height of a few inches below the side arm. A collodion tube attached to the tapered end of the small tube was held in

place by the adapter. The rubber stopper was fitted on the large glass tube so that the collodion tube was submerged in the buffer and the small glass tube was open to the air. Then enzyme solution was added to the collodion tube, by means of the small tube, to the height of the buffer. The large glass tube was placed in an ice-water bath. A vacuum was applied using a water aspirator. The protein, in about 0.2 ml. of solution, was concentrated at the bottom of the collodion tube. Eight ml. of solution could be concentrated in an hour.

d. Evaporation in a dialysis tube. The enzyme solution was placed in a dialysis bag in a cold room. An electric fan directed air against the dialysis bag until the solution reached the desired volume.

e. Sephadex. Sephadex G-25, coarse, was added to the enzyme solution until the suspension became thick. It was stirred for 10 minutes to insure complete swelling. The resin was allowed to settle and a disk of filter paper placed over it. It was centrifuged and the supernatant retained. The filter cake was washed two or three times with buffer. The above is the method of Flodin (60).

f. Aquacide I and II. The enzyme solution was dialyzed against a viscous solution of 15% Aquacide I or 7.5% Aquacide II. The Aquacide concentrated the enzyme solution by dehydration.

The concentrated enzyme solution resulting from the above methods was dialyzed against 0.1 M phosphate buffer, pH 7.2 for 12 hours.

6. Ammonia determination

For the measurement of ammonium sulfate in effluent fractions from Sephadex columns a modification of the procedure of Hawk, Oser and Summerson was used (80). For the measurement of ammonia production by the enzyme the procedure of Johnson was used (81). After stopping the enzyme reaction using trichloroacetic acid, one ml. of the supernatant was added to 2 ml. of distilled water, 2 ml. of Nesslers solution No. 2 and 5 ml. of 1.0 N sodium hydroxide, shaking between each addition. The solution was allowed to stand 15 minutes to develop the color and the optical density was read at 490 mμ against a denatured enzyme blank.

7. Phosphate determination

Phosphate was determined by the method of Sudo et al. (82). Four ml. of sample solution were mixed with 0.4 ml. of molybdenum reagent. Then 0.4 ml. of freshly prepared hydroquinone solution (0.050 gm. in 10 ml. of distilled water) was added and this solution was warmed in a water bath at 50-60^{o}C for 15 minutes. The optical density of the cooled sample was determined at 660 mμ against a reagent blank.

8. Amino acid determination

Amino acids were determined by the method of Moore and Stein (83). To 0.1 ml. of sample was added 1.0 ml. of ninhydrin solution. The resulting solution was heated for 20 minutes in a boiling water bath. Five ml. of diluent (1:1 of n-propanol and water) were added and the solution was allowed to stand 15 minutes. The optical density was read at 570 mμ against a reagent blank.

9. Disc gel electrophoresis

The procedure recommended by Canal Industrial Corporation was followed. Premixed standard gels were polymerized in 5 mm. i.d. x 70 mm. cylindrical glass tubes. First a small pore, lower gel was polymerized in which the electrophoretic separation actually takes place. Next a large pore, spacer gel was polymerized in which the proteins are sorted according to their mobilities into highly concentrated discs before reaching the lower gel. Finally, a large pore, upper gel containing the sample was polymerized. The ends of the vertical column were immersed in tris-glycine buffer, pH 8.6 and a current of 4 ma. per column was maintained for 30-45 minutes. The gel was removed from the column and immediately stained unless stated otherwise. For protein determinations, the stain was amido black. After destaining electrophoretically, the columns were stored in 10% acetic acid.

C. Purification Procedure

The purification was carried out at 2°C whenever possible. Centrifugations were carried out using an International high speed refrigerated centrifuge, Model HR-1 at 10,000 rpm. for 20 minutes. However, whenever cloudy samples were obtained, a Servall SS-1 centrifuge at 20,000 x g was used for 30 minutes, to remove the cloudiness.

1. Activity test

Each liver was tested rapidly by adding 60 ml. of 0.1 M phosphate buffer, pH 7.2 to 15 gm. of ground liver and homogenizing for 30 seconds. The remainder of the liver was frozen immediately. The homogenate was assayed and those livers containing 20 units per gm. or above were selected for purification.

2. Homogenate (41)

Fresh or thawed, fresh frozen, sheep liver was washed with cold distilled water, ground in a hand meat grinder and homogenized in a waring blendor for 45 seconds with twice its volume of 0.1 M phosphate buffer, pH 7.2. The purity index of this homogenate was used as the starting level and all expressions of purification were based on this value.

3. First heat treatment

The crude homogenate was poured into a four liter beaker and heated to 70°C in a boiling water bath, with

stirring (about 15 minutes). Immediately upon attainment of this temperature, the homogenate was cooled in an ice-water bath to 20°C (41). The heat treated material was filtered through muslin into a large beaker and the precipitate extracted once with 1/10 the original volume. The filtration required about 5 hours.

4. First ammonium sulfate fractionation (41)

The solution was made 40% saturated with ammonium sulfate at 25°C by adding 243 gm. salt per liter of solution. Stirring was achieved using a magnetic stirrer. At this point the pH was adjusted to 7.2 by the addition of concentrated ammonia. After standing at 2°C for at least four hours, the solution was filtered with a Hormann filter using a D-3 filter pad. Enough Celite Hydroflow filter aid was added to form a half-inch pad. The filtrate was retained and brought to 55% saturation with ammonium sulfate by adding 97 gm. salt per liter of solution. The precipitate was collected after allowing the solution to stand overnight. It was then dissolved in 1/5 the original volume of 0.1 M phosphate buffer, pH 7.2. The amount of ammonium sulfate used was taken from a table in Methods of Enzymology, p.76 (84).

5. Second heat treatment (41)

The solution was brought to 70°C in a boiling water bath (about 5 minutes) and was then transferred to a

constant temperature bath at 70°C and maintained at this temperature for 10 minutes. A magnetic stirrer was used. The precipitate was discarded.

6. Second ammonium sulfate fractionation

The heat treated solution was made 30% saturated with ammonium sulfate at 25°C by adding 176 gm. salt per liter of solution. Then the pH was adjusted to 7.2 with concentrated ammonia. This solution was allowed to stand at least five hours. The precipitate was discarded and the clear supernatant was brought up to 50% saturation with ammonium sulfate by the addition of 127 gm. salt per liter of solution. It was allowed to stand overnight. The precipitate was collected and dissolved in 10-25 ml. of 0.1 M phosphate buffer, pH 7.2.

7. Preparation of DEAE-Sephadex gel

Dry DEAE-Sephadex powder, A-50 medium, was suspended in dilute phosphate buffer pH 7.2 and stirred a few minutes to allow swelling of the gels. After sedimentation the fines were removed by decantation. Additional phosphate buffer was added and the decantation was repeated until all the fines were removed. Then the gel was filtered using a Buchner funnel and washed with 100 ml. of 0.25 N hydrochloric acid followed by water. The neutral gel was placed in 0.5 N sodium hydroxide and stirred for 15 minutes. Afterwards, it was filtered and washed with water. Finally,

it was placed in the appropriate buffer, usually 0.025 M phosphate buffer, pH 7.2 and decanted several times.

8. Packing the column

A column, 2 x 36 cm., fitted at the bottom with a stopcock was filled with buffer. A small piece of glass wool was placed over the outlet capillary followed by a 2 cm. layer of glass beads. The top of the column was connected to a large funnel. Buffer was added until it reached the neck of the funnel, care being taken to avoid air bubbles. The prepared DEAE-Sephadex gel was decanted and added to the funnel. An air driven stirrer was used to agitate the gel. Buffer was allowed to flow through the column at a rate of 40 ml./hr. When the gel reached the bottom of the column, the flow was stopped to allow a 3 cm. bed to form. Afterwards the flow was started again until a height of 28 cm. was obtained. The funnel and connecting tube were removed and a disk of filter paper with a diameter slightly smaller than the bore of the column was placed on the bed. The column was placed in a cold room, at 2°C and buffer was run through the column overnight to stabilize the bed. This packing procedure is similar to the one of Flodin (55).

In later experiments, satisfactory columns were prepared by pouring a slurry of DEAE-Sephadex into the column tube. The column was filled with buffer and a small piece

of glass wool was placed over the outlet capillary followed by a 2 cm. layer of glass beads. The buffer was drained to about 1/5 the height of the column. A slurry of DEAE-Sephadex was added and a layer of a few centimeters was allowed to form. Then a flow rate of 40 ml./hr. was started. More of the slurry was added when about 75% of the gel had settled. This addition was repeated until the gel column reached the desired height.

9. Preparation of the sample

The enzyme solution from the second ammonium sulfate fractionation was dialyzed against two one-liter changes of 0.025 M phosphate buffer, pH 7.2 for 24 hours. Any precipitate resulting from dialysis was removed by centrifugation.

In later experiments, the residual ammonium sulfate was removed and the phosphate buffer concentration was changed from 0.100 M to 0.025 M by passing the enzyme solution through a 2.5 x 62 cm. Sephadex G-25, coarse, column with 0.025 M phosphate buffer, pH 7.2. This column was packed by adding a slurry of Sephadex G-25. A maximum of 20 ml. of enzyme solution could be passed through the column in 30 minutes. A two fold dilution usually occurred, however this was no major problem since DEAE-Sephadex concentrated the protein.

10. Chromatography on DEAE-Sephadex

All runs were carried out in a cold room at 2°C. Ten ml. fractions were collected using a fraction collector with either a drop counter or volume counter. A 2.5 x 28 cm. DEAE-Sephadex A-50, medium, column was prepared. Satisfactory results were obtained using samples of 100 to 750 mgm. protein. The sample was added to the column in 5 ml. aliquots and washed onto the column with an equivalent volume of 0.025 M phosphate buffer, pH 7.2 also added in 5 ml. aliquots. The protein appeared to be adsorbed at the top of the resin bed indicated by the formation of a yellow band. A stepwise elution was carried out using phosphate buffers of 0.05, 0.10 and 0.50 M concentration, pH 7.2. A flow rate of 45 ml./hr. was maintained.

IV. RESULTS

A. Activation Attempts

1. Initial activity

Russell et al. reported that the sheep liver L-threonine dehydrase could be activated by conversion of a protein precursor into the enzyme (85). They obtained a 20 fold increase in activity by incubating the enzyme homogenate at 45°C for two hours at pH 9.0. However, attempts to repeat this in our laboratory were unsuccessful.

A preliminary investigation of the effect of various proteolytic enzymes on the initial activity was undertaken. Liver homogenates were treated with carboxypeptidase, chymotrypsin, papain and trypsin for five minute and thirty minute periods at 37°C. At the end of each period, aliquots were assayed following the regular assay procedure. The results revealed that no activation had occurred as shown in Table 1.

2. Effect of α-keto acids

The effect of α-keto acids on the enzyme activity was determined by following the production of ammonia. Sodium pyruvate, sodium α-ketobutyrate, sodium α-ketovalerate and α-ketoglutaric acid were adjusted to neutral pH and 0.3 ml. of each was incubated with 0.6 ml. of enzyme for 15 minutes, at pH 7.2, at 37°C. This mixture was incubated with 0.3 ml.

Table 1. The effect of proteolytic enzymes on the L-threonine dehydrase in sheep liver homogenates

Agent 6 mgm.	Incubation[a] time	Units[b]	% Initial activity
None	0	1.90	100
Carboxypeptidase	5	1.37	73
Carboxypeptidase	30	1.43	76
Chymotrypsin	5	0.97	51
Chymotrypsin	30	0.54	29
Papain	5	1.25	66
Papain	30	0.61	32
Trypsin	5	0.44	26
Trypsin	30	0.24	13
Trypsin & inhibitor[c]	30	1.88	99

[a]Time of incubation of liver homogenates with the proteolytic enzyme at 37°C.

[b]Micromoles of α-ketobutyrate produced per hour at 37°C, pH 8.9.

[c]Trypsin soybean inhibitor.

of 0.5 M tris-carbonate buffer, pH 8.95 and 0.3 ml. of buffered substrate, pH 8.9 for one hour at 37°C. The reaction mixtures contained 1×10^{-5} M and 1×10^{-3} M of added α-keto acid. The reaction was stopped by the addition of 0.5 ml. of 25% trichloroacetic acid. One ml. was used for

the determination of ammonia by the method of Johnson (81). The ammonia production of samples containing no added α-keto acid agreed with the production of α-ketobutyrate by these samples. No activation occurred. At 1 X 10^{-3} M, the α-keto acids inhibited the reaction. Table 2 shows the effect of the addition of pyruvate and α-ketobutyrate on the enzyme activity.

B. Purification Procedure

The present purification procedure is a modification of the procedure of Davis and Metzler and is superior in terms of the purification yielded and the time involved. The modifications which were made include the following:

1. The method of collecting the precipitate from the first heat treatment was changed in order to decrease the time required for this step. In working up large batches of liver, 3 or more livers, filtration through muslin was used instead of centrifugation. The filtration process required only two hours compared to at least 5 hours for centrifugation. In addition the filtration process required less attention. One disadvantage of the filtration process was that not all of the enzyme solution was recovered. However, this loss was minimized by washing the precipitate with buffer and filtering it again. Therefore, the entire filtration process required about 4 hours. Whenever less

Table 2. The effect of α-keto acids on the enzyme activity[a]

Substrate	α-Keto acid added		Product		% Activity
		Concentration			
L-Threonine	------	------	18.00[b]	18.20[c]	100
L-Threonine	Pyruvate	1×10^{-5} M		17.70[c]	98
L-Threonine	Pyruvate	1×10^{-3} M		4.12[c]	23
L-Threonine	α-Ketobutyrate	1×10^{-5} M		17.70[c]	98
L-Threonine	α-Ketobutyrate	1×10^{-3} M		9.00[c]	50
L-Serine	------	------	2.52[b]	2.50[c]	100
L-Serine	Pyruvate	1×10^{-5} M		1.87[c]	75
L-Serine	Pyruvate	1×10^{-3} M		0.56[c]	22
L-Serine	α-Ketobutyrate	1×10^{-5} M		2.06[c]	82
L-Serine	α-Ketobutyrate	1×10^{-3} M		0.81[c]	32

[a]To 0.6 ml. of enzyme was added 0.3 ml. of α-keto acid and incubated 15 minutes before the regular assay procedure.

[b]Micromoles of α-keto acid produced per hour at 37°C, pH 8.9.

[c]Micromoles of ammonia produced per hour at 37°C, pH 8.9.

than three livers were worked up at one time, centrifugation was just as rapid as filtration through muslin and the recovery of enzyme was better.

2. In the procedure of Davis and Metzler precipitation from a 40% saturated ammonium sulfate solution was performed after the second heat treatment. However a sizeable portion of the enzyme was not precipitated under these conditions. In addition, much of the precipitate was unwanted protein. Therefore, a pilot fractionation was performed to determine the concentration of ammonium sulfate most suitable for the precipitation of the enzyme. Aliquots of enzyme solution were made 30, 40, 45, 50, 55, and 60% saturated with ammonium sulfate. The resulting precipitates were centrifuged and redissolved in an equal volume of phosphate buffer, pH 7.2. The results of this fractionation revealed that the fraction which was precipitated between 30-50% ammonium sulfate saturation should be retained.

3. No appreciable purification resulted from a third heat treatment; therefore, it was eliminated from the procedure.

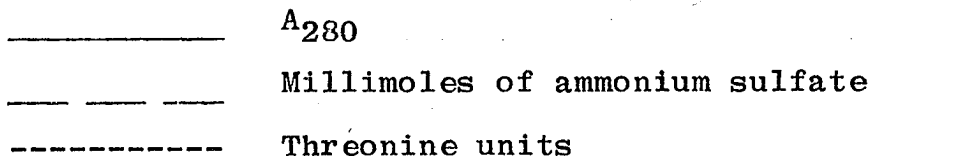
4. Chromatography on Sephadex G-25 was added to the purification procedure. This addition decreased the time required to change the phosphate buffer concentration and to remove the ammonium sulfate remaining after the second ammonium sulfate fractionation; chromatography on Sephadex

G-25 required only 30 minutes whereas dialysis required 24 hours. In addition chromatography on Sephadex G-25 usually increased the purity index of the enzyme. A typical elution pattern of the chromatography of L-threonine dehydrase on Sephadex G-25 is shown in Fig. 2. At least 90% of the protein and the units were recovered.

5. Chromatography on DEAE-Sephadex also was added to the purification procedure. The stepwise elution of the enzyme from DEAE-Sephadex is shown in Fig. 3. A small yellow band, fraction I was eluted from the column at 0.10 M phosphate buffer, pH 7.2. A second yellow band, fraction II was eluted at 0.50 M phosphate buffer, pH 7.2. A small amount of protein could not be removed from the gel. At least 61% of the protein and 77% of the units could be recovered.

Fractions I and II, both contained enzyme activity towards L-threonine and L-serine and neither was activated by pyridoxal phosphate, adenylic acid, adenosine diphosphate or glutathione. Fraction I contained approximately 9% of the protein and 48% of the units, which had been added to the column. This was equivalent to a 5-10 fold purification of the enzyme contained in fraction I over the previous step. Fraction II contained 5-33% of the protein and 5-25% of the units placed on the column; equivalent to a 2 fold purification.

Fig. 2. The elution pattern of the chromatography of L-threonine dehydrase on Sephadex G-25.



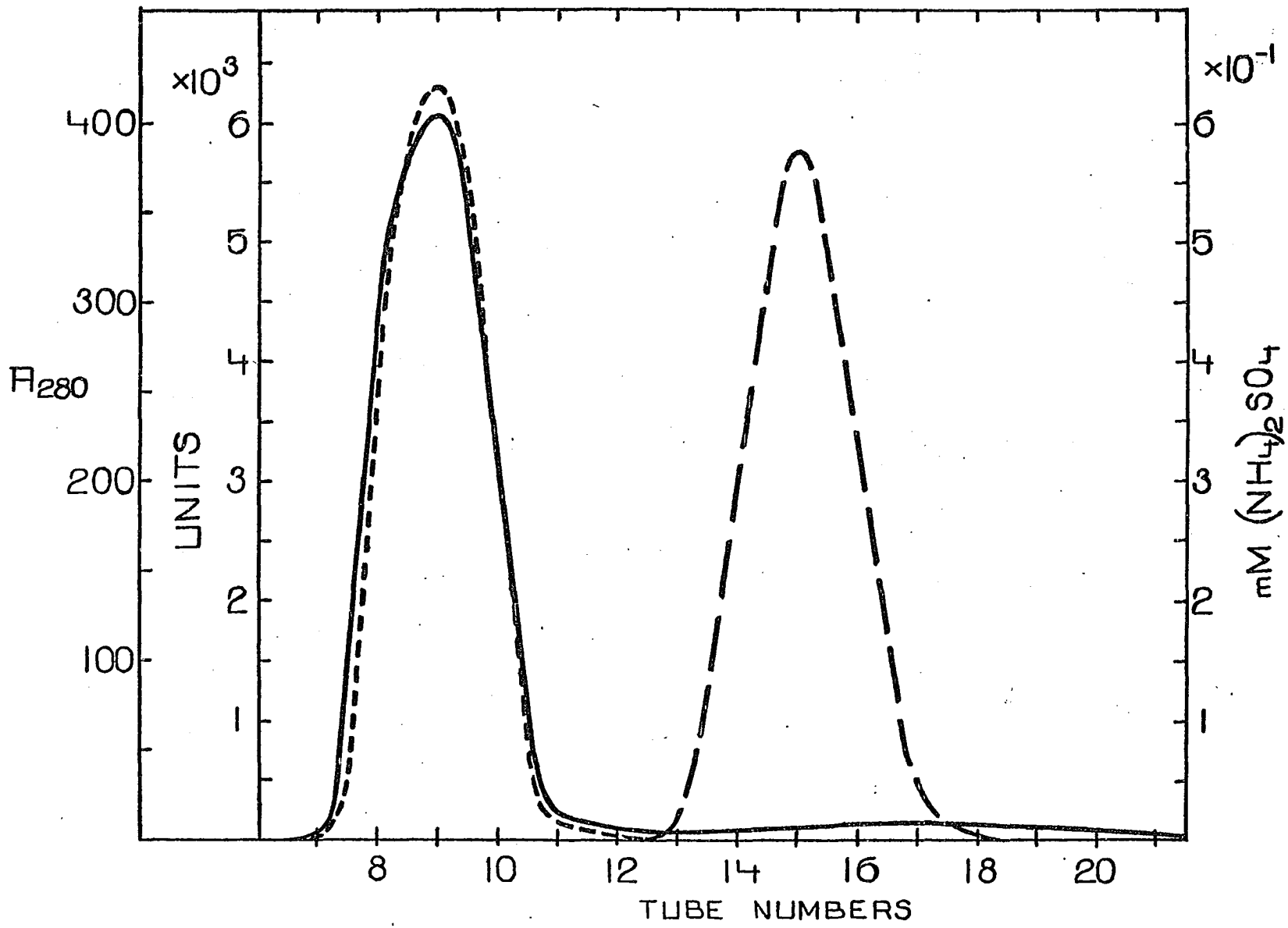
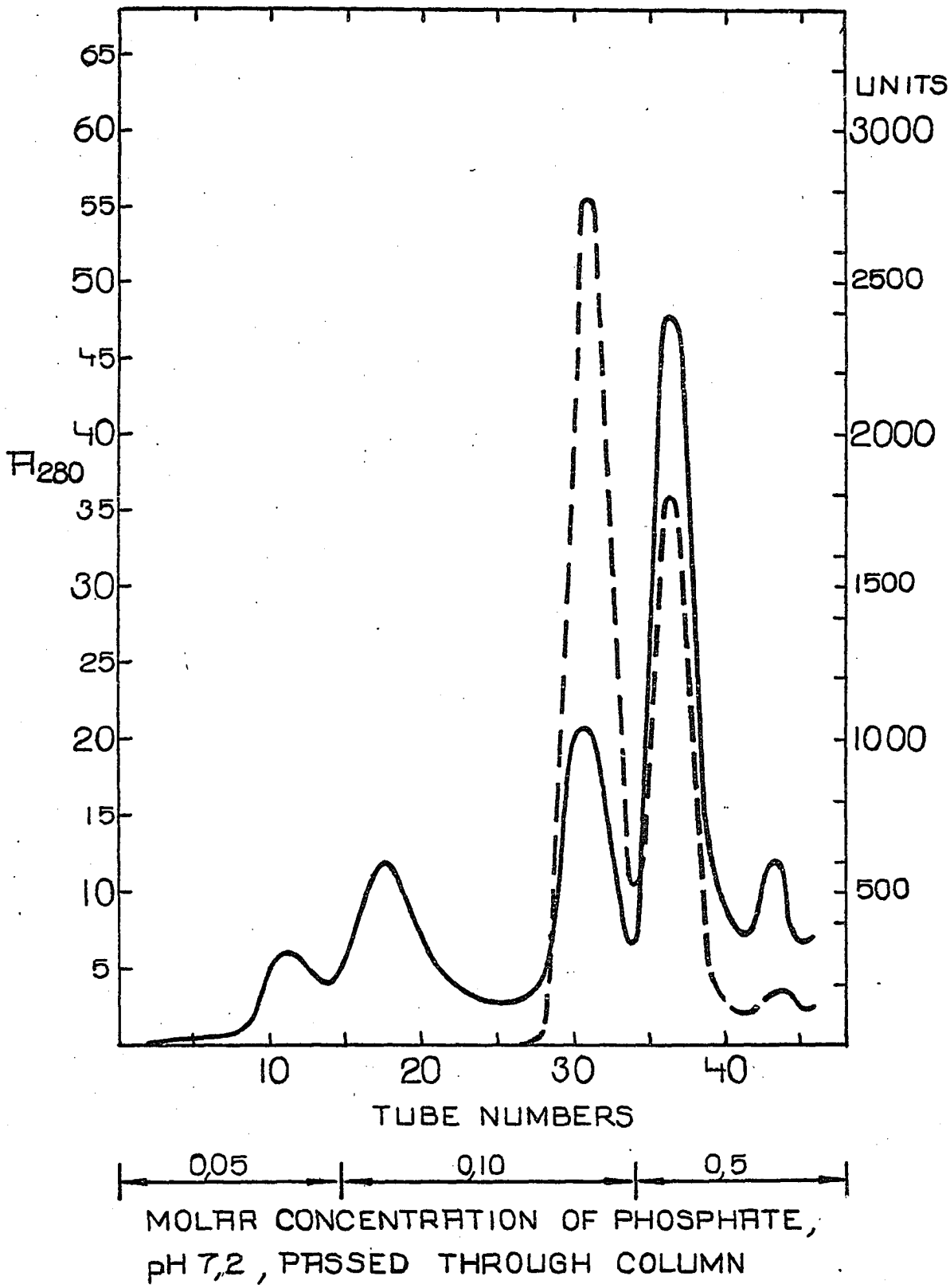

400
300
200
100
A_{280}
$\times 10^3$
6
5
4
3
2
1
UNITS
$\times 10^{-1}$
6
5
4
3
2
1
mM $(NH_4)_2SO_4$
8
10
12
14
16
18
20
TUBE NUMBERS

Fig. 3. The elution pattern of the chromatography of L-threonine dehydrase on DEAE-Sephadex

———— $A_{280\ m}$

---------- Threonine units


65
60
55
50
45
40
35
30
25
20
15
10
5
F_{280}
UNITS
3000
2500
2000
1500
1000
500
10
20
30
40
TUBE NUMBERS
0,05
0,10
0,5
MOLAR CONCENTRATION OF PHOSPHATE,
pH 7,2 , PASSED THROUGH COLUMN

Therefore, one step was added, two steps were improved and one step was eliminated to give a procedure consisting of two heat treatments, two ammonium sulfate fractionations and chromatography on DEAE-Sephadex. Table 3 shows the step by step results of the purification of a typical batch of L-threonine dehydrase. A final purity index of 300-600 units per mgm. protein was usually obtained. However, final purity indicies as high as 1600 units per mgm. protein have been obtained but not consistently. Of fifteen preparations, two had purity indices above 1300 units per mgm. protein and four had purity indices above 500 units per mgm. protein. The remainder had purity indices below 500 units per mgm. protein. Table 4 shows the range of variation in the purification of L-threonine dehydrase.

The final purity index depended on the initial concentration of enzyme in the sheep liver to a large extent. This concentration varied greatly. Nishimura reported enzyme activities from zero to 160 units per gm., measured at pH 7.2 (32). The enzyme is less active at pH 7.2 compared to pH 8.9 (32,40,41). Davis reported enzyme activities from zero to 13 units per ml., measured at pH 8.9 (40,41). Our results, measured at pH 8.9, indicate enzyme activities from zero to 75 units per gm. corresponding to a range in units per ml. of crude extract from zero to 32. No correlation could be made between the livers and their

Table 3. The step by step results of the purification of L-threonine dehydrase

Fraction	Volume ml.	Protein mgm.	Units[a]	Purity[b] index	Fold[c] pure	Yield %
Homogenate	1,900	345,000	55,000	0.175	1	100
First heat treatment	1,010	57,000	55,500	0.970	5	101
First $(NH_4)_2SO_4$ fractionation	520	4,950	62,000	12.50	72	112
Second heat treatment	520	3,400	54,500	16.00	92	99
Second $(NH_4)_2SO_4$ fractionation	26	740	47,500	64.00	365	87
Chromatography on DEAE-Sephadex[d]						
Fraction I	30	17	7,520	445.00	2,500	25
Fraction II	40	41	3,180	80.00	450	9

[a]Micromoles of α-ketobutyrate produced per hour at 37°C, pH 8.9.

[b]Units of enzyme per mgm. protein.

[c]Fold purification over purity index of homogenate.

[d]Used only 16 ml. of enzyme solution from second $(NH_4)_2SO_4$ fraction.

Table 4. The range of variation in the purification of the L-threonine dehydrase of sheep liver.

Fraction	Purity index	Fold purification	Yield %
Homogenate	0.06 - 0.18	1	100
First heat treatment	0.63 - 1.49	4 - 10	57 - 216
First $(NH_4)_2SO_4$ fractionation	7.30 - 12.5	50 - 110	39 - 112
Second heat treatment	8.32 - 22.1	55 - 126	45 - 99
Second $(NH_4)_2SO_4$ fractionation	22.00 - 130	143 - 350	44 - 90
Chromatography on DEAE-Sephadex			
Fraction I	289 - 1,680	893 - 19,700	3 - 30
Fraction II	62 - 164	370 - 2,500	6 - 29

initial concentration of enzyme; however we have observed that lamb livers often contain less enzyme than mature sheep livers. Those livers containing 20 units per gm. or above were selected for purification. Usually 3-5 livers were selected at a time. They could be worked up through the second ammonium sulfate fractionation in 3 days.

C. Methods of Concentrating Enzyme

The enzyme contained in fractions I and II from the chromatography on DEAE-Sephadex was concentrated for further studies. The protein concentration of these fractions was usually low; the optical density at 280 mμ was between 0.10 and 0.80. Therefore, a series of methods were investigated to determine the most efficient method of concentrating the enzyme. The methods were judged on the basis of the following criteria:

1. The purity index should not decrease.
2. The total units should not decrease.
3. Any volume desired should be attainable.
4. A minimum time should be required.

Of the methods shown in Table 5, the use of ammonium sulfate, 60% or saturated, and the use of vacuum filtration best satisfied the above criteria. Each method recovered all of the activity with very little loss of the total units. The concentrate was a precipitate in the case of the ammonium

Table 5. Methods of concentrating enzyme[a]

Method	Volume Before	Volume After	Time hrs.	P[b] mgm.	Units[c]	Purity index	P[b] %	Units %	Activity %
Vacuum filtration	50	5	10	93	1460	16	61	81	127
Saturated $(NH_4)_2SO_4$	25	3.2	2.5	62	277	45	67	80	120
60% $(NH_4)_2SO_4$	50	10	5	25	2860	114	80	82	102
Sephadex	25	9	0.5	12	86	7	56	58	103
Evaporation	50	24	12	36	3400	97	114	98	87
Aquacide I	25	6	47	14	308	22	147	89	61
Aquacide II	25	13	47	11	272	25	119	79	66

[a]In all of the above methods, the concentrated enzyme was dialyzed against two liters of 0.1 M phosphate buffer, pH 7.2 for 12 hours.

[b]P: protein

[c]Micromoles of α-ketobutyrate produced per hour at 37°C, pH 8.9.

sulfate or a volume of about 0.2 ml. in the case of vacuum filtration. Therefore, each concentrate could be adjusted to any desired volume. A maximum of five hours was required in the case of ammonium sulfate. Vacuum filtration concentrated at a rate of 8 ml./hr. Each method had its operational advantages. Therefore, vaccum filtration was used for small volumes, 25 ml. or less. Dialysis against saturated ammonium sulfate was used for volumes up to 100 ml. Larger volumes were concentrated by the method of precipitation from a 60% saturated ammonium sulfate solution.

D. Purity of Enzyme

The purity of the enzyme was studied on the basis of its sedimentation and electrophoretic mobility. Those enzyme preparations used for these studies had purity indices of 300-500 units per mgm. protein. Although more active preparations were available, their protein concentration was too low to give satisfactory results. Attempts to concentrate these very active preparations were unsuccessful. It should be pointed out that no very active preparations have been obtained since the results of the methods for concentrating the enzyme were available.

1. Ultracentrifugation

Fraction I from the chromatography on DEAE-Sephdex was concentrated and dialyzed against two changes of 0.05

M phosphate buffer, pH 7.2 for 24 hours. The homogeneity of this preparation was determined by centrifuging it at 59,780 rpm. in a Model E Spinco ultracentrifuge. A protein concentration of at least 5 mgm. per ml. of solution was necessary for satisfactory results. Three peaks were observed in the schlieren pattern, as shown in Fig. 4. Although these peaks are not sharp, approximate sedimentation constants were calculated by the following method (86):

$$s = \frac{\frac{dx}{dt}}{\omega^2 x}$$

$$\frac{dx}{x} = s\omega^2 dt$$

$$\text{Log } x = \frac{s\omega^2 t}{2.303}$$

s = sedimentation constant in Svedberg units (10^{-13} sec.)

x = distance from the center of rotation in cm.

t = time in min.

ω = angular velocity of the centrifuge in radians per sec.

When Log x vs t is plotted, the slope equals $\frac{s}{F}$. F is the value $\frac{\omega^2}{2.303}$ for each speed and is equal to 9.79×10^{-10} for 59,780 rpm. Approximate sedimentation constants were calculated from three different ultracentrifuge runs, as shown in Table 6.

Fig. 4. The schlieren pattern of the L-threonine dehydrase of sheep liver with a purity index of 405 units per mgm. protein. Centrifuged at 59,780 rpm. in a Model E Spinco ultracentrifuge. Pictures were taken at 8 minute intervals.

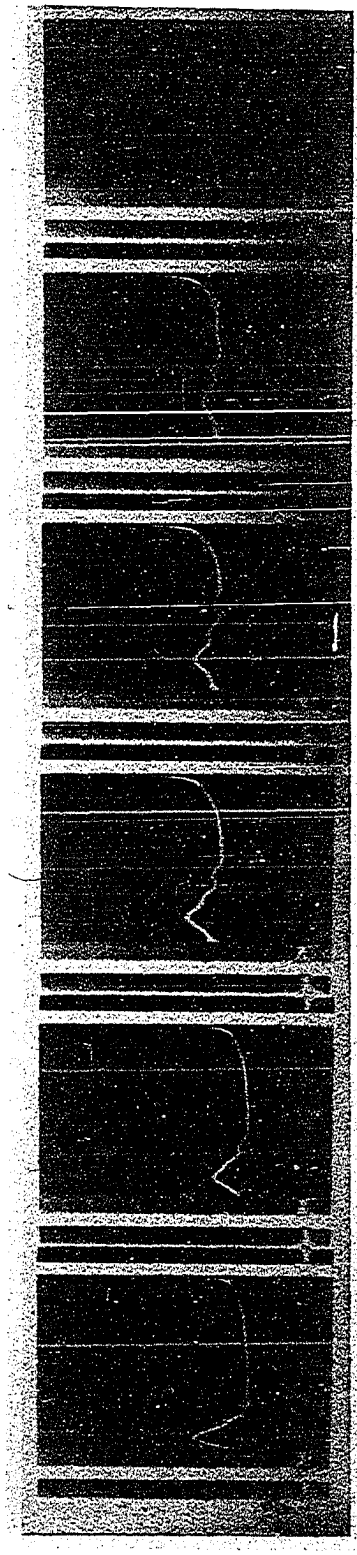


Table 6. Sedimentation constants of L-threonine dehydrase after chromatography on DEAE-Sephadex[a]

Purity index	$Peak_1$	$Peak_2$	$Peak_3$
210	None	6.30	11.75
310	4.75	8.60	11.50
405	5.15	8.80	12.00

[a]Ultracentrifuge runs were made at 59,780 rpm. in a Model E Spinco ultracentrifuge. The values are given in Svedberg units (10^{-13} sec.).

Approximate molecular weights of the components were determined by three different methods. In the first method, the proteins were assumed to be spherical and 30% hydrated. An equation showing the relationship between the molecular weight and the sedimentation constant is shown below. Corrections had to be made for the hydration of the protein and they are also shown below (87).

$$M = \frac{Nfs}{(1-\bar{v}\rho)}$$

$$\frac{f}{f_o} = \left(1 + \frac{w}{\bar{v}\rho}\right)^{\frac{1}{3}} = \phi$$

$$f_o = 6\pi\eta r$$

$$r = \left(\frac{4\ M\bar{v}}{3\pi N}\right)^{\frac{1}{3}}$$

$$M^{\frac{2}{3}} = \frac{Ns\phi 6\pi\eta\left(\frac{4\bar{v}}{3N\pi}\right)^{\frac{1}{3}}}{1-\bar{v}\rho}$$

M = molecular weight

N = Avogadro's number

s = sedimentation constant

$\bar{v}$ = partial specific volume

ρ = density of water

f = frictional coefficient (hydrated)

f_o = frictional coefficient (anhydrous)

w = hydration of protein

η = viscosity

r = radius of protein

In the second method used to determine the molecular weight, a plot was made of the sedimentation constant vs molecular weight of several well characterized proteins. The data was obtained from a table in The Proteins, p. 634 (87). The molecular weight was obtained from a smooth curve drawn through the points. In the third method, the same assumptions were made as in the first method. The molecular weight was obtained from a nomogram in The Proteins, p. 666 (87). Table 7 contains the molecular weights.

2. Disc gel electrophoresis

Fig. 5 shows the disc gel electrophoresis pattern of the enzyme at various stages of the purification. The protein concentration varied from step to step making comparisons difficult. In addition as the purification proceeded, new protein bands appeared as they became more concentrated. Nevertheless, it is possible to observe the

Table 7. The molecular weight of the proteins in the enzyme solution after chromatography on DEAE-Sephadex

Sedimentation constant	Molecular weight X 10^{-3}		
	Equation	Graph	Nomogram
4.75 - 5.15	65 - 75	68 - 77	60 - 70
8.60 - 8.80	158 - 164	172 - 180	110 - 150
11.50 -11.75	244 - 261	272 - 290	225 - 250

the disappearance of protein bands and the appearance of new bands as the enzyme is purified.

In order to locate the enzyme on the gel columns, after electrophoresis the gel columns were incubated with L-threonine at 37°C for 20 minutes. The gel columns were placed in 0.5% 2,4-dinitrophenylhydrazine solution and a yellow band formed in the top portion of the column within one hour. However, if the column was allowed to remain in the 2,4-dinitrophenylhydrazine solution overnight, it reacted with the polyacrylamide gel, making the results ambiquous and doubtful. The diffusion of α-ketobutyrate out of the gel further complicated this staining procedure. A fluorometric method for the determination of α-keto acid also was tried using o-phenyldiamine (88). However the high concentration of sulfuric acid dehydrated the gel.

Fig. 5. Disc gel electrophoresis patterns of L-threonine dehydrase at various stages of purification.

A: homogenate

B: 1st heat treatment

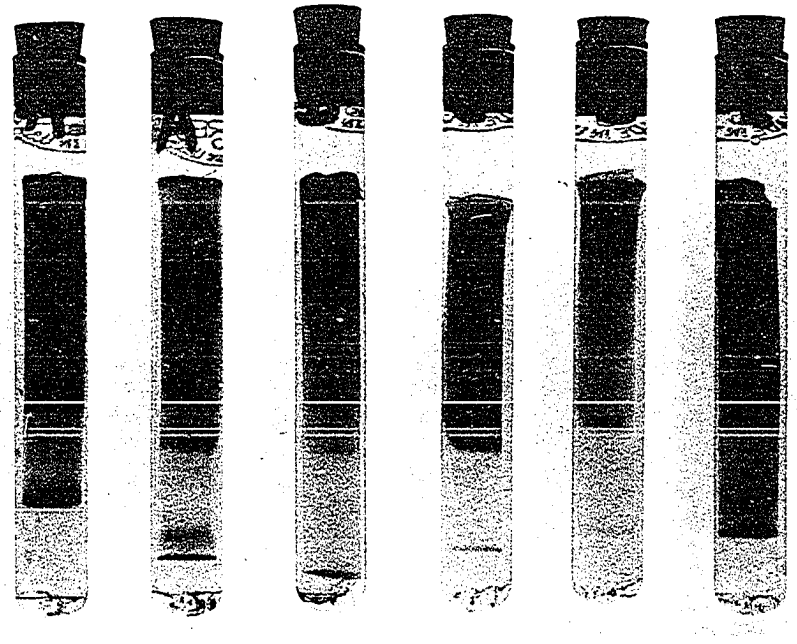
C: 1st ammonium sulfate fractionation

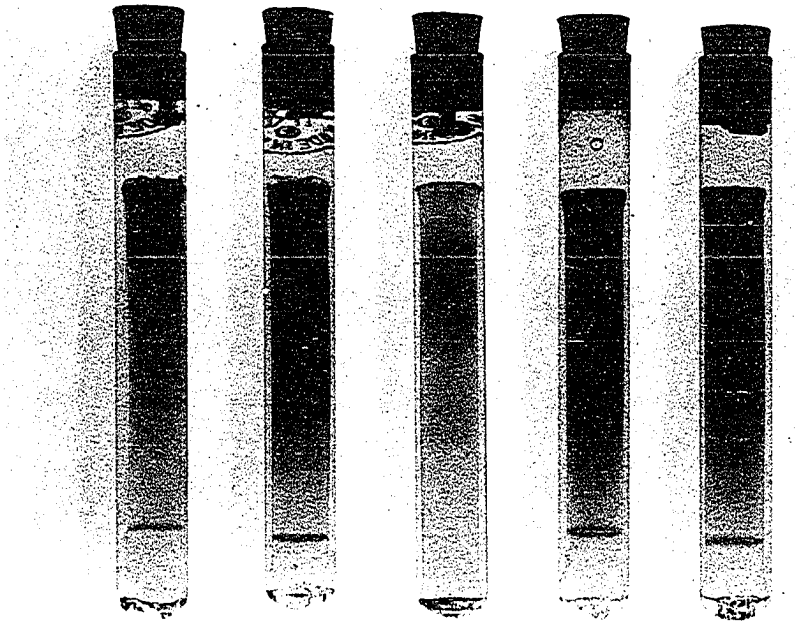
D: 2nd heat treatment

E: 2nd ammonium sulfate fractionation

I: fraction I from chromatography on DEAE-Sephadex

II: fraction II from chromatography on DEAE-Sephadex



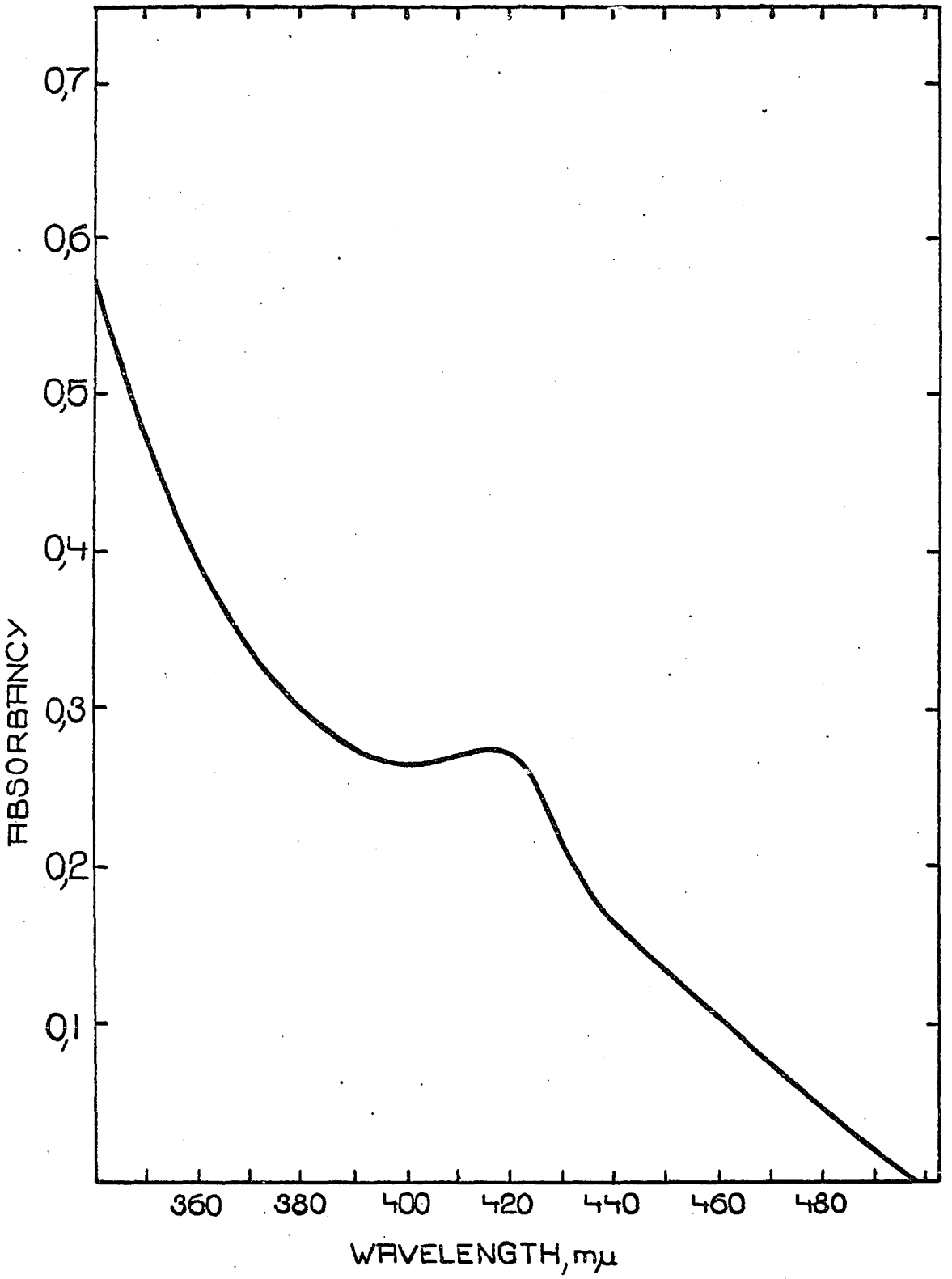


The general location of the enzyme was determined by slicing the unstained gel columns, horizontally, into six equal sections. Each section was placed in one ml. of 0.1 M phosphate buffer, pH 7.2 and disrupted using a wooden applicator. After standing overnight, the supernatant was decanted and incubated with L-threonine for 30 minutes. The addition of 0.5% 2,4-dinitrophenylhydrazine, 95% ethanol and 2.5 N sodium hydroxide provided a qualitative test for the production of α-ketobutyrate. Again the enzyme appeared to be located in top portion of the gel.

3. Spectrum

The complete spectrum of fraction I and II, from the chromatography on DEAE-Sephadex (260 mμ to 500 mμ) revealed two peaks. One peak absorbs at 278 mμ, and corresponds to the aromatic amino acids. The other peak, as shown in Fig. 6, absorbs at 405 mμ to 410 mμ. This peak may represent bound pyridoxal phosphate. More evidence is needed to prove this though. The absorbancy in the 400 mμ region possesses less than 10% of the absorbancy of the peak at 278 mμ. Occassionally the 405 mμ to 410 mμ peak is observed in less active preparations.

Fig. 6. The spectrum of L-threonine dehydrase between 350 mμ and 500 mμ.


0,7
0,6
0,5
0,4
0,3
0,2
0,1
ABSORBANCY
360
380
400
420
440
460
480
WAVELENGTH, mμ

E. Stability of Enzyme

Chromatography on DEAE-Sephadex required about 30 hours. During part of this time, the concentration of enzyme was very low. After chromatography, the enzyme occassionally, remained at room temperature several hours while preparations were made for the assay. In order to insure that no loss of enzyme occurred when the concentration of enzyme was low or when the enzyme was retained at room temperature for a considerable period of time, the stability of the enzyme was determined under these conditions.

It was observed that dialysis of the enzyme against distilled water resulted in the loss of enzyme activity. Therefore, the stability and activity of the enzyme under variable conditions of ionic environment was also determined.

1. Effect of temperature

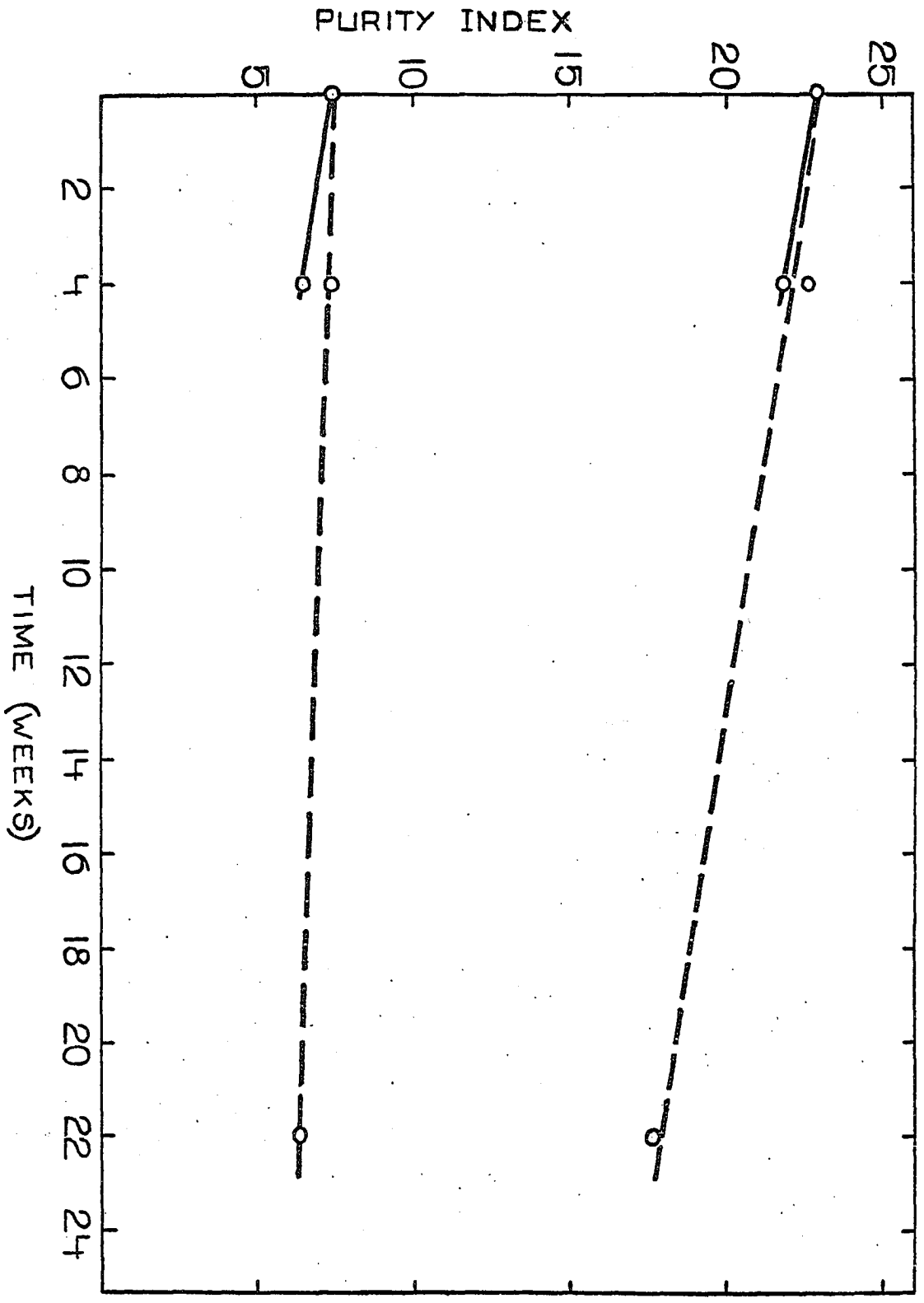
Preliminary studies were carried out to determine the stability of the enzyme frozen, refrigerated, and at room temperature as a function of time.

a. Frozen. The enzyme solution was divided into two portions. One portion was frozen and the other portion refrigerated. Periodic determinations of the purity index were made. As shown in Fig. 7, the enzyme is more stable in the frozen state. The more active fraction lost activity at a faster rate. However, very little change occurred

Fig. 7. The stability of L-threonine dehydrase as a function of time when frozen and refrigerated. The purity index is based on the total units and total protein.

_____ Frozen enzyme

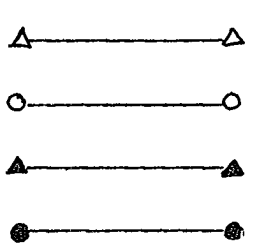
---------- Refrigerated enzyme


PURITY INDEX
5
10
15
20
25
TIME (WEEKS)
2
4
6
8
10
12
14
16
18
20
22
24

in the activity of both samples within one month. Therefore, it is satisfactory to store the enzyme in the refrigerator during regular use. Some precipitate, believed to be bacterial growth, formed in the enzyme samples and was removed by centrifugation.

b. Room temperature. Again, the enzyme solution was divided into two portions. One portion was refrigerated and the other stored at room temperature (about 25°C). Each portion was stored both as a concentrated enzyme solution and as a diluted enzyme solution (1/100). The diluted enzyme solutions were stored in test tubes, each containing 0.9 ml. of enzyme solution. Periodic determinations of the purity index were made. As shown in Fig. 8, all the samples are stable for at least 12 hours. As expected, the concentrated enzyme solutions retain their activity for a longer period of time. The refreigerated diluted samples are of special interest because this is the condition of the enzyme eluted from the column. After 36 hours, the refrigerated samples maintained their activity almost as well as the refrigerated concentrated samples. Since the concentration of enzyme is low for only about 15 of the 30 hours required for chromatography, very little, if any, enzyme activity is lost during the chromatography on DEAE-Sephadex resulting from dilution.

Fig. 8. The stability of L-threonine dehydrase as a function of time when refrigerated and stored at room temperature (about 25°C).

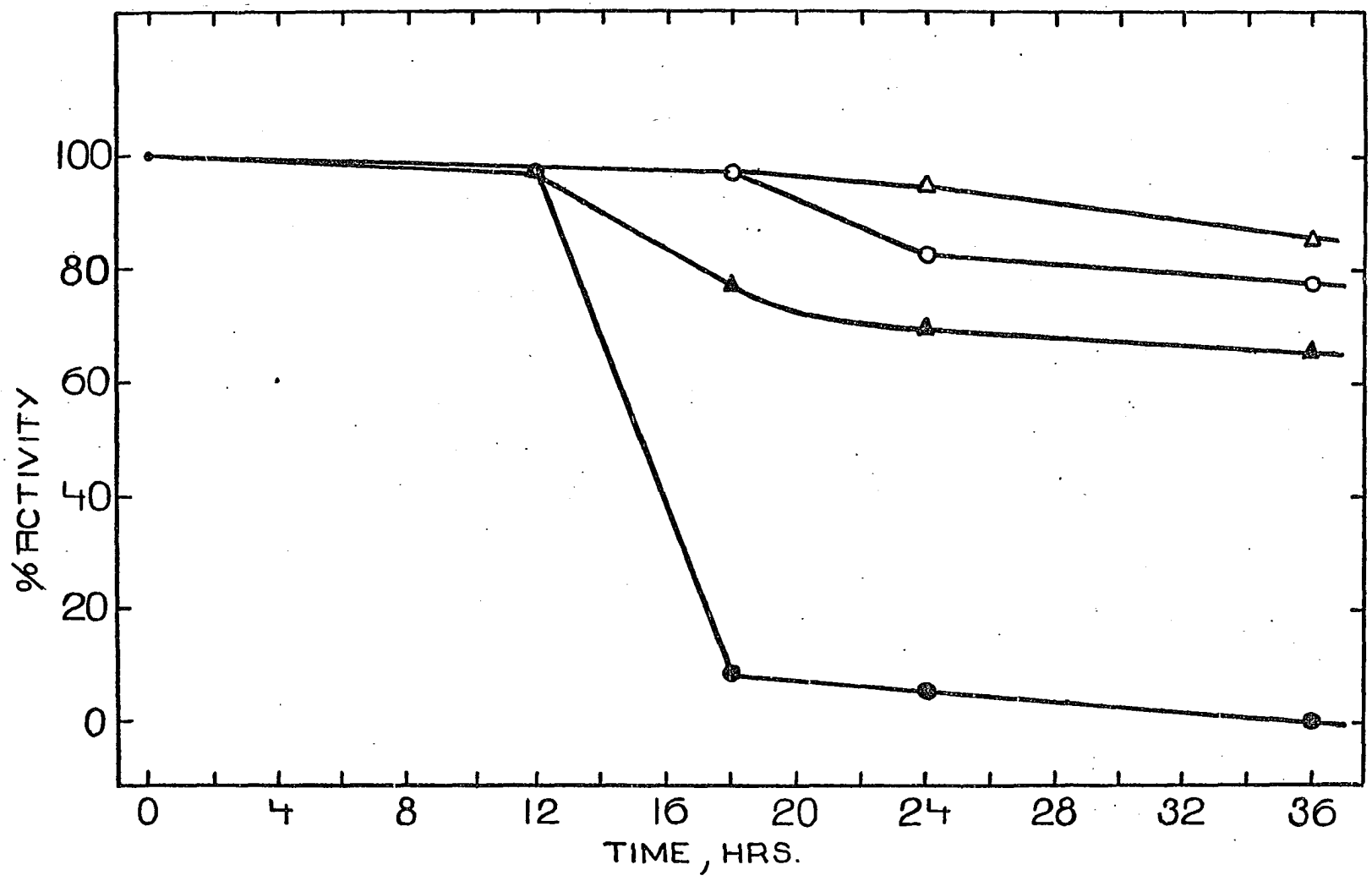


Concentrated refrigerated enzyme

Diluted refrigerated enzyme

Concentrated enzyme at room temperature

Diluted enzyme at room temperature


100
80
60
40
20
0
% ACTIVITY
0
4
8
12
16
20
24
28
32
36
TIME, HRS.

2. Effect of ionic environment

The effect of the ionic environment was studied by desalting the enzyme by means of chromatography and adding various reagents to this desalted enzyme to note their effect. Similar experiments were run with enzyme diluted to an extent that the original environment was negligible.

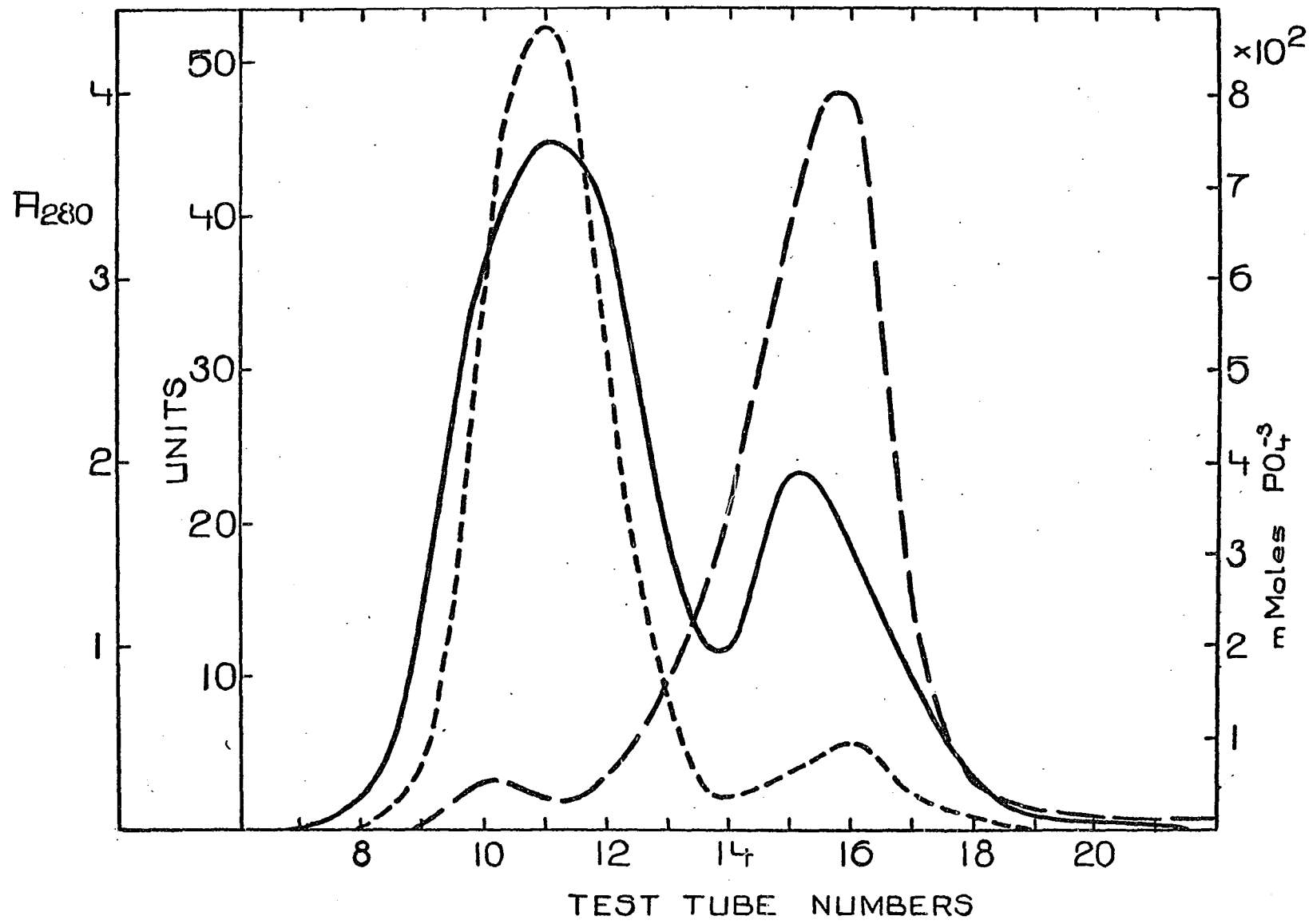
a. Desalting of enzyme. The enzyme was desalted by chromatography on either Sephadex G-25, coarse, column, or ion retardation resin, AG11A8, column. Therefore a Sephadex G-25, coarse, column, 2.0 x 92 cm. and an ion retardation column, 2.0 x 82 cm. were prepared using deionized, distilled water. Three ml. of enzyme solution containing 415 to 2550 units were added to either column and eluted with redistilled water. Four ml. fractions were collected. The time required to complete the chromatography varied from 3 1/2 hours in initial experiments to 15 minutes in later experiments. This variation in the time required for chromatography did not affect the efficiency of the column. A typical elution pattern is shown in Fig. 9. This particular elution pattern is from the chromatography on Sephadex G-25. However chromatography on ion retardation resin gave a similar elution pattern. A small amount of potassium was still associated with the enzyme after chromatography. This association was dependent on the initial potassium phosphate concentration to a small degree; for example samples

Fig. 9. The elution pattern of the desalting of L-threonine dehydrase.

———————— A_{280}

—— —— —— Millimoles of potassium phosphate

---------- Threonine units

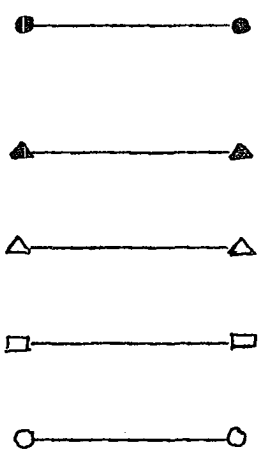

A280
UNITS
×10²
mMoles PO4-3
TEST TUBE NUMBERS
8
10
12
14
16
18
20

in 0.1 M potassium phosphate had 6 X 10^{-4} M still associated after chromatography and samples in 0.025 M potassium phosphate had 2 X 10^{-4} M still associated. For stability determinations the enzyme in tubes 9-12 from the Sephadex G-25 column or 18-22 from the ion retardation column was used; it contained very little potassium phosphate and the bulk of the desalted enzyme.

b. Stability of desalted enzyme. Immediately following the elution of the desalted enzyme, solutions used in this section were prepared in the following manner. To 4.5 ml. of desalted enzyme was added 0.5 ml. of each additive. For studies on the desalted enzyme, 0.5 ml. of redistilled water was added to the desalted enzyme. The solutions were assayed in the usual manner.

The stability of the desalted enzyme is shown in Fig. 10. The enzyme was added to the column at zero time. A dashed line is drawn from zero time to the time of the first assay, indicating that the shape of the curve during this period is not known. As illustrated, the enzyme is rapidly inactivated in the presence of low salt concentration. This inactivation appears to be affected by the time required to elute the desalted enzyme from the column. Otherwise, desalted enzyme solutions with the same concentration of potassium phosphate would be inactivated to the same extent but they are not. Instead, as the time required for

Fig. 10. The stability of the desalted enzyme as a function of time. The dashed line represents the time from the addition of the enzyme solution to a Sephadex G-25 column until the time of the first assay. The shape of the curve during this time is not known.



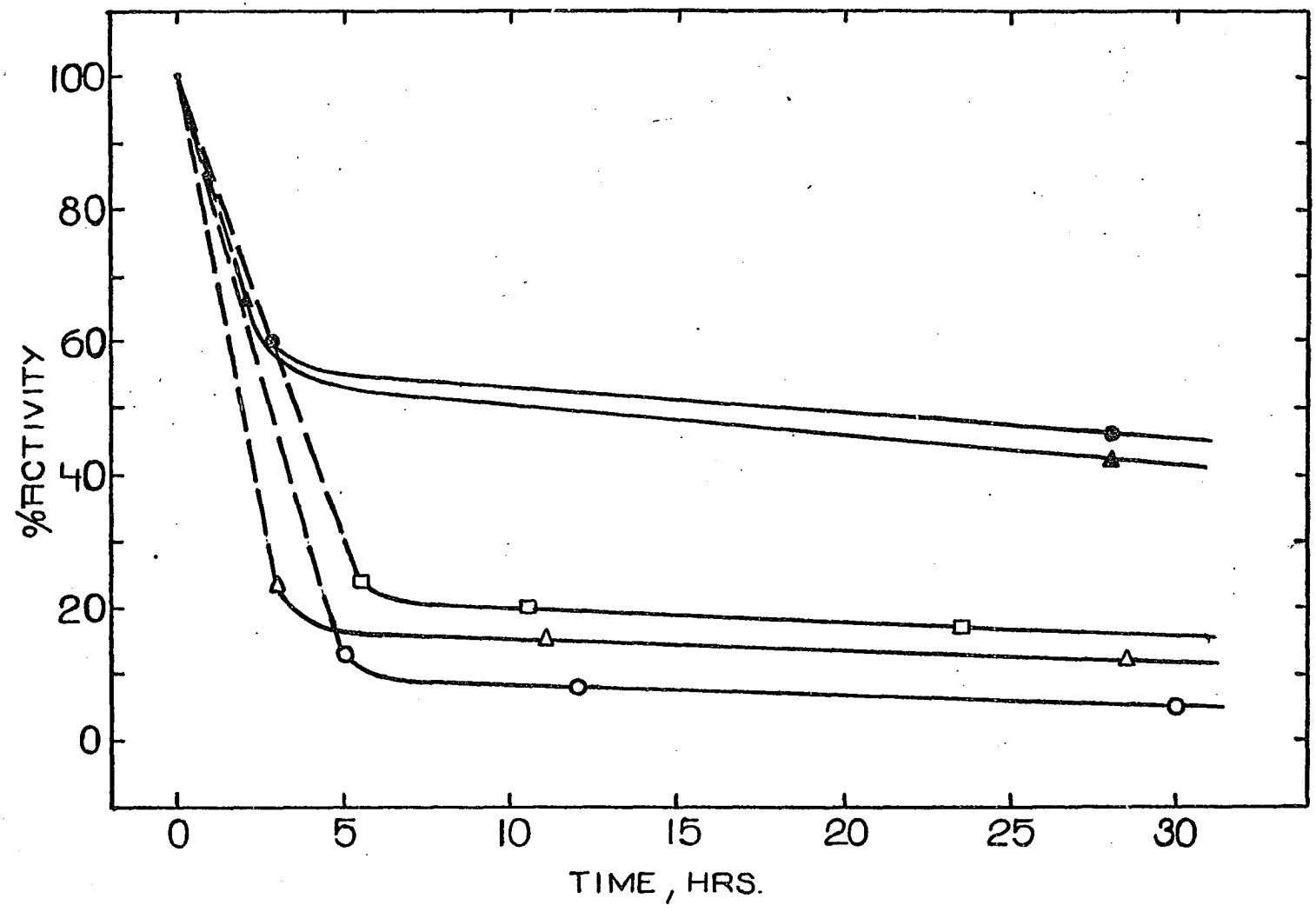
Required 15 minutes for chromatography.
2×10^{-4} M potassium phosphate and 1×10^{-2} M mercaptoethanol still associated.

Required 15 minutes for chromatography
1×10^{-4} M potassium phosphate still associated.

Required 2 hours for chromatography.
6×10^{-4} M potassium phosphate still associated.

Required 3.5 hours for chromatography.
25×10^{-4} M potassium phosphate still associated.

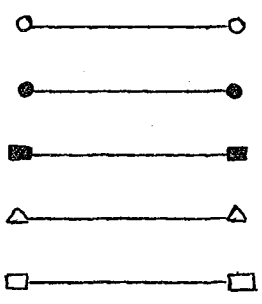
Required 4 hours for chromatography.
3×10^{-4} M potassium phosphate still associated.


100
80
60
40
20
0
%ACTIVITY
0
5
10
15
20
25
30
TIME, HRS.

elution increased, the inactivation increased. The minimum inactivation obtained was 34%, when the desalted enzyme was eluted in 15 minutes.

Solutions were also prepared which were 5×10^{-3} M with respect to potassium phosphate, potassium chloride and ammonium sulfate. Their effect is shown in Fig. 11. The time required to elute the desalted enzyme from the column is depicted by the portion of the dashed line forming the hypotenuse of the triangles. The additives were added at least 1/2 hour before the assay was made. Therefore a dashed line is drawn from the time of addition of the salt to the time of the assay. It should be pointed out that the desalting experiments yielded no evidence to support the reactivation implied by the dashed line; however, the results of dilution studies support the reactivation. All of the salts inhibited the rate of inactivation. In addition, potassium phosphate appears to have reactivated the desalted enzyme. At 5×10^{-2} M potassium phosphate, the apparent reactivation is more pronounced. The apparent reactivation was also affected by the time required to elute the desalted enzyme from the column; since the greater the inactivation the less the apparent reactivation. The highest apparent reactivation occurred when the desalted enzyme was eluted in 15 minutes and 66% of the initial activity was retained. The addition of potassium phosphate,

Fig. 11. The effect of salts on the desalted enzyme as a function of time. Two experiments are illustrated. The time required to elute the column is denoted by the portion of the dashed line forming the hypotenuse of the triangles. The second dashed line illustrates the time of addition of the salt to the time of the first assay.



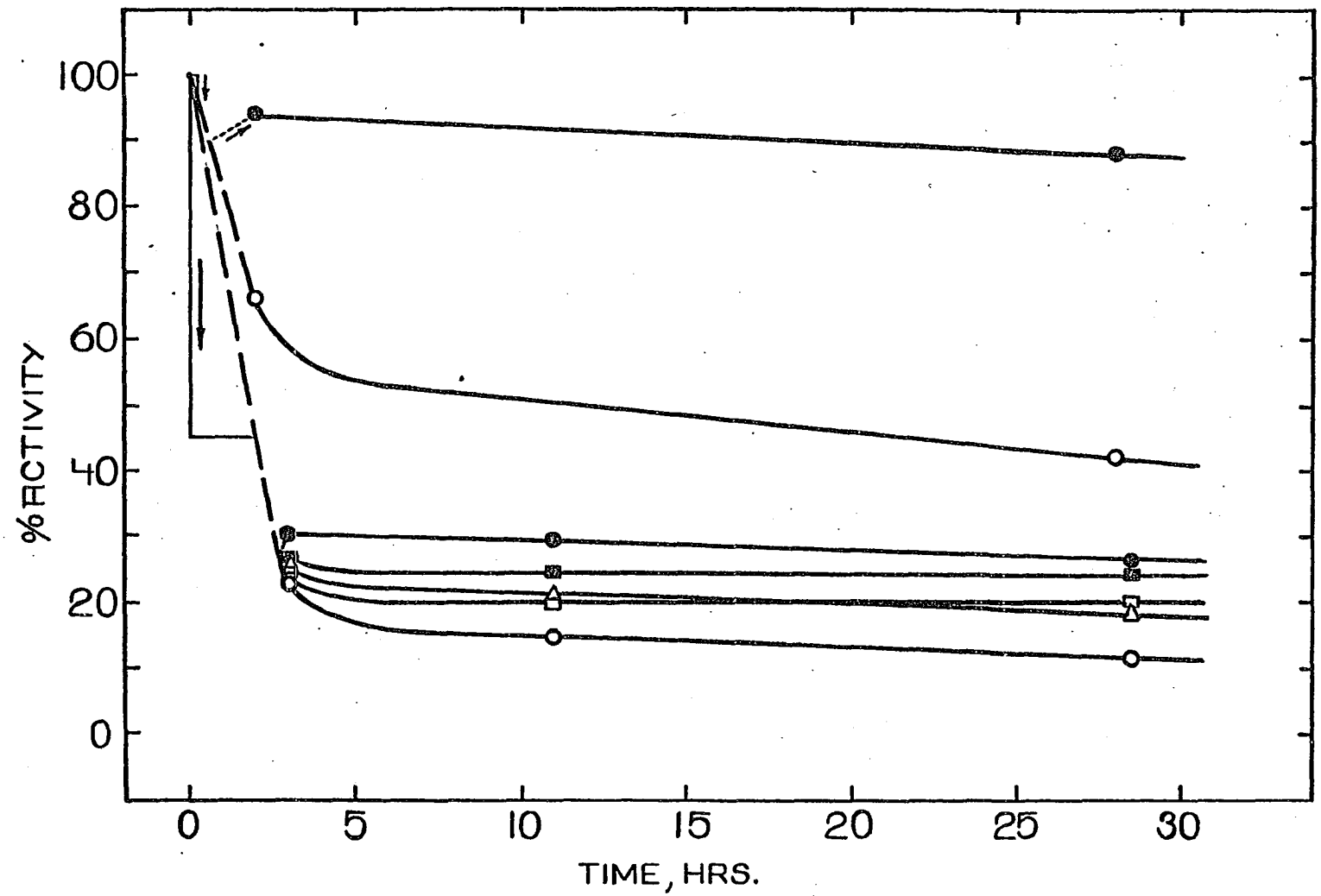
Desalted enzyme

Desalted enzyme in 5 X 10^{-2} M potassium phosphate

Desalted enzyme in 5 X 10^{-3} M potassium phosphate

Desalted enzyme in 5 X 10^{-3} M potassium chloride

Desalted enzyme in 5 X 10^{-3} M ammonium sulfate


100
80
60
40
20
0
% ACTIVITY
0
5
10
15
20
25
30
TIME, HRS.

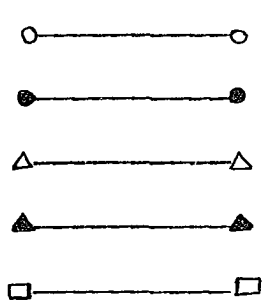
3×10^{-3} M, "reactivated" the enzyme to 93% of its original activity.

Solutions of pyridoxal phosphate and adenosine diphosphate, 1×10^{-4} M and of mercaptoethanol, 1×10^{-3} M were also prepared. Their effect is shown in Fig. 12. Pyridoxal phosphate and mercaptoethanol stabilized the desalted enzyme; however, no apparent activation was observed. Adenosine diphosphate was ineffective.

Since mercaptoethanol retards the inactivation of the desalted enzyme and is not a salt, an attempt was made to utilize this effect to protect the enzyme during the desalting process. In this way, it would be possible to retard or eliminate the initial inactivation. Initial inactivation refers to the rapid inactivation taking place as the enzyme is desalted on the column. Therefore, a Sephadex G-25, coarse, column was prepared using 1×10^{-2} M mercaptoethanol. The enzyme was precipitated using ammonium sulfate and dissolved in 0.025 M phosphate buffer, pH 7.2 containing 1×10^{-2} M mercaptoethanol. This enzyme solution was added to the column and eluted with 1×10^{-2} M mercaptoethanol in 15 minutes. As shown in Fig. 10, compared to a enzyme solution desalted in the absence of mercaptoethanol, the mercaptoethanol does not inhibit the initial inactivation of the enzyme.

All of the previous results on the stability of the

Fig. 12. The effect of additives on the desalted enzyme as a function of time. The chromatography required 3.5 hours. The dashed line represents the time from the addition of the enzyme solution to a Sephadex G-25 column until the time of the first assay. The shape of the curve during this time is not known.



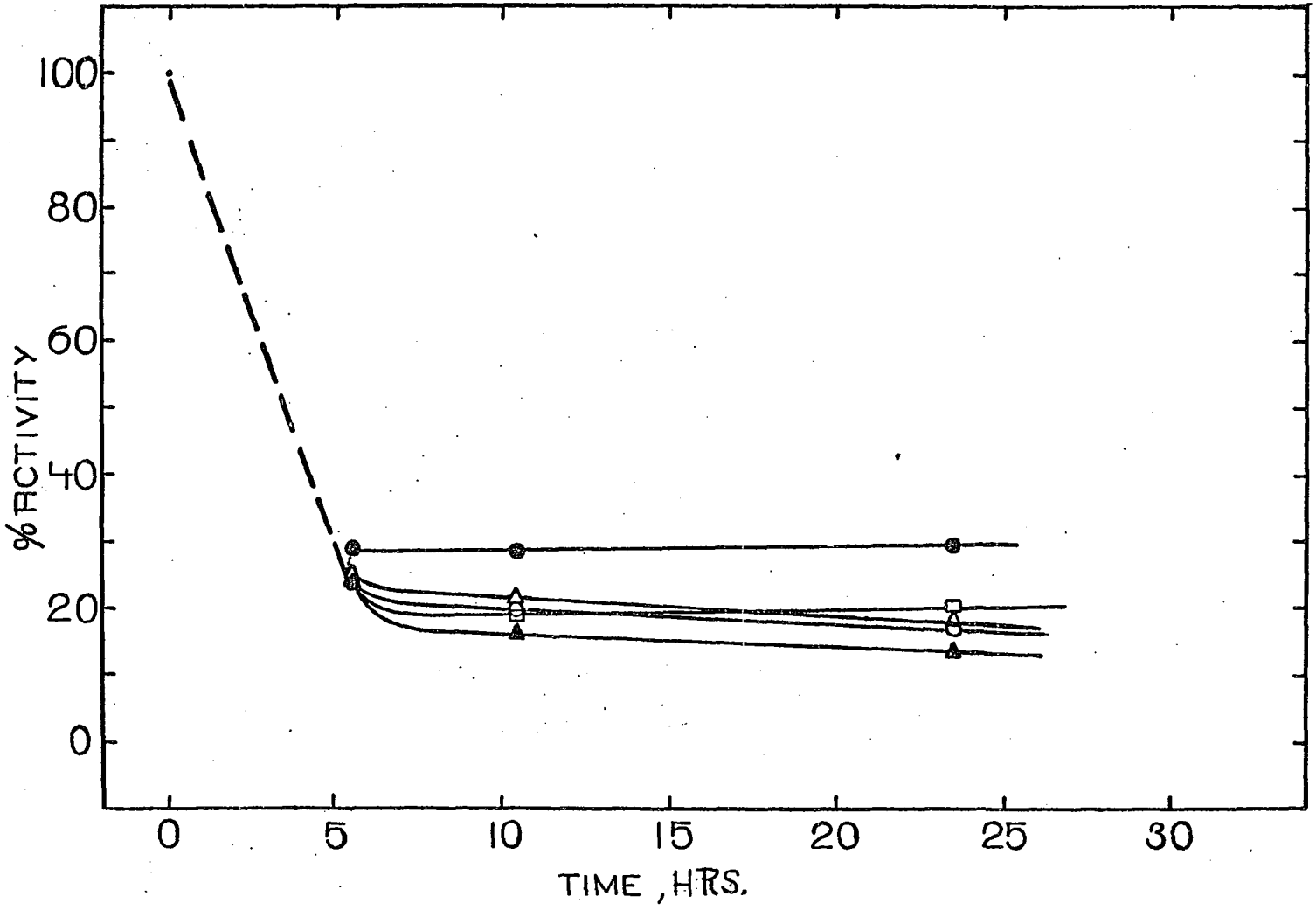
Desalted enzyme

Desalted enzyme in 5×10^{-2} M potassium phosphate

Desalted enzyme in 1×10^{-4} M pyridoxal phosphate

Desalted enzyme in 1×10^{-4} M adenosine diphosphate

Desalted enzyme in 1×10^{-3} M mercaptoethanol


100
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20
0
% ACTIVITY
0
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20
25
30
TIME , HRS.

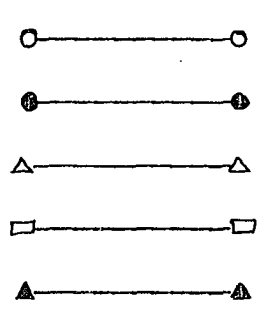
desalted enzyme was from enzyme desalted by means of Sephadex G-25. When the enzyme was desalted by means of the ion retardation column, similar results were obtained as shown in Fig. 13.

Although the study of the effect of the ionic environment on the enzyme is not complete, the methods and techniques used up to this point were discontinued. The techniques were unsatisfactory because they did not allow sufficient control of the experiments. In addition, the information attainable was limited by these methods. The disadvantages of the desalting methods are as follows:

1. Chromatography on either Sephadex G-25 or ion retardation resin did not completely remove all of the potassium phosphate from the enzyme solution; however, the concentration was low enough to affect the activity of the enzyme. It was impossible to predetermine how much potassium phosphate would remain in the enzyme solution after the chromatography. In order to compare the effects of various salts, the ionic environment of the enzyme must be constant.

2. It was possible that the initial inactivation of the enzyme in a low concentration of potassium phosphate was actually caused by the column and not directly caused by the ionic environment, especially since it appears that the extent of the inactivation was affected by the length of time required to elute the desalted enzyme from the column.

Fig. 13. The effect of salts on the desalted enzyme, prepared from an ion retardation column, as a function of time. The time required to elute the desalted enzyme from the column is shown by the portion of the dashed line forming the hypotenuse of the triangle. The second dashed line illustrates the time of addition of the salt to the time of the first assay.



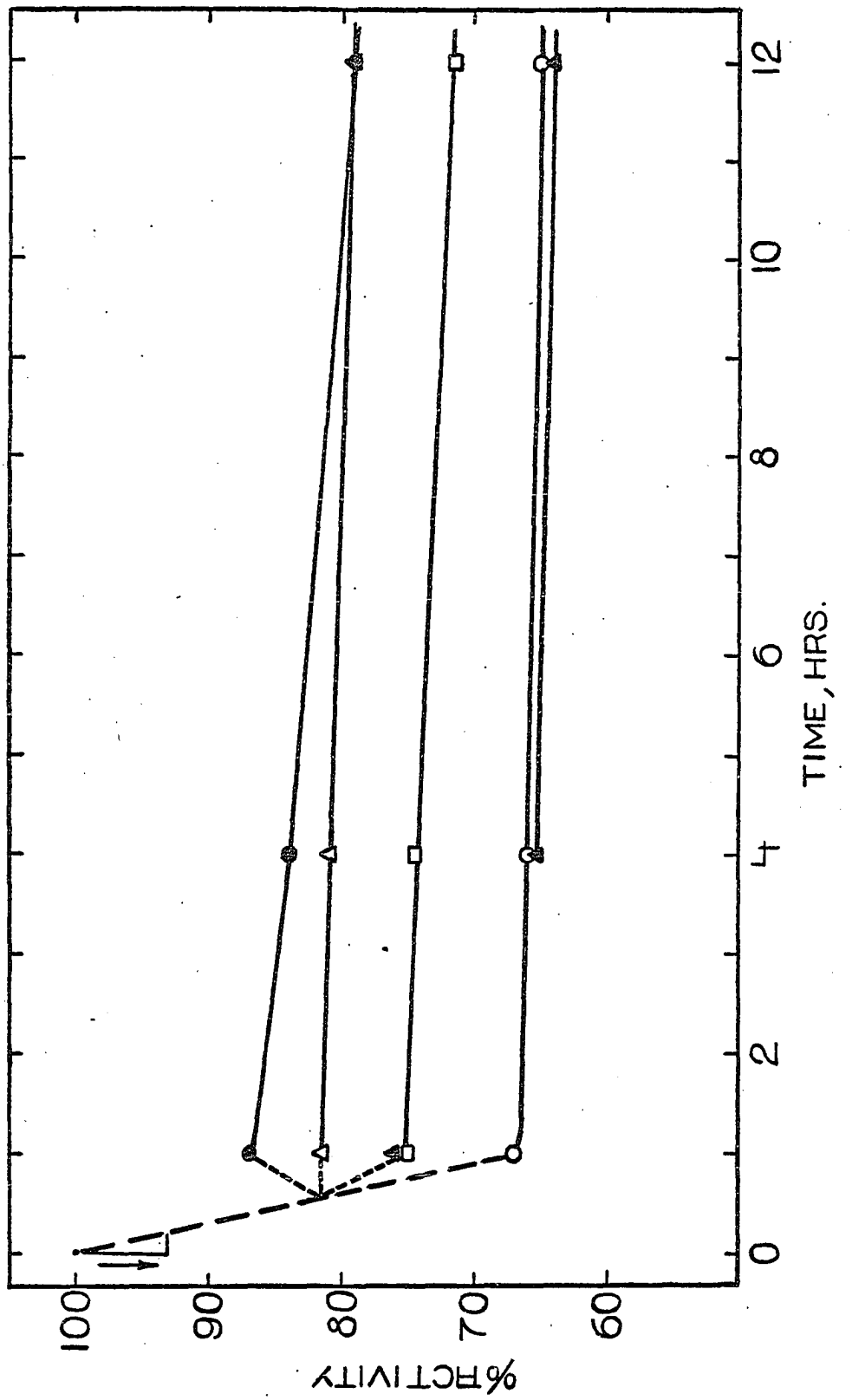
Desalted enzyme

Desalted enzyme in 1 X 10^{-3} M potassium phosphate

Desalted enzyme in 1 X 10^{-3} M potassium chloride

Desalted enzyme in 1 X 10^{-3} M ammonium sulfate

Desalted enzyme in 1 X 10^{-2} M mercaptoethanol


%ACTIVITY
100
90
80
70
60
0
2
4
6
8
10
12
TIME, HRS.

3. If the initial inactivation does result from low salt concentration, it would be interesting to know the shape of the curve during the initial inactivation.

4. One of the most interesting questions raised by the desalting experiments is does the addition of potassium phosphate actually reactivate the desalted enzyme? Since the shape of the curve for the initial inactivation is not known and since the potassium phosphate was added at least 1/2 hour before the assay was taken, it is possible that the potassium phosphate is only stabilizing the enzyme from further inactivation.

3. Dilution studies

In order to answer the questions raised by the desalting experiments, the potassium phosphate concentration was reduced by means of dilution. The enzyme was concentrated and dialyzed for 24 hours against two changes of 0.10 M phosphate buffer, pH 7.2.

a. Effect of potassium phosphate on enzyme. The concentrated enzyme solution was diluted 1/1,000 with distilled water or phosphate buffer to give the desired potassium phosphate concentration and assayed immediately in the usual manner. Table 8 shows the activity of the enzyme in the presence of various concentrations of potassium phosphate. It clearly indicates that the enzyme is less active in a low ionic environment. The activity remaining

in the samples, at the same potassium phosphate concentration, obtained by means of either dilution or desalting in 15 minutes agreed very well.

Table 8. The activity of the enzyme in various concentrations of potassium phosphate[a]

KHP[b] conc.	% Activity		
	0, hrs.[c]	2, hrs.[c]	12, hrs.[c]
1×10^{-1} M	100	100	100
9×10^{-3} M	100	100	97
1×10^{-3} M	86	85	73
1×10^{-4} M	65	62	41
1×10^{-5} M	49 - 59	---	---
5×10^{-6} M	37 - 49	---	---

[a]Enzyme solution was diluted 1/1,000 with distilled water or 0.1 M potassium phosphate, pH 7.2. The solutions were assayed in the usual manner.

[b]KHP: potassium phosphate.

[c]Length of time after the dilution of the enzyme.

b. Reversal of the inactivation of enzyme in a low ionic environment. In order to show that the addition of potassium phosphate reverses the inactivation resulting from a low ionic environment, the following experiment was performed. The concentrated enzyme solution was dialyzed

24 hours against two changes of 0.025 M phosphate buffer, pH 7.2. Therefore, solutions diluted 1/1,000, 1/2,500 and 1/5,000 with distilled water corresponded to a concentration of 2.5×10^{-5} M, 1×10^{-5} M and 5×10^{-6} M potassium phosphate respectively. Portions of these solutions were adjusted immediately to 0.1 M potassium phosphate by the addition of 2 ml. of 0.5 M potassium phosphate to 8 ml. of the enzyme solution. As a reference, the concentrated enzyme solution was diluted as above, using 0.1 M potassium phosphate. All of the above solutions were immediately assayed for 15 minutes in the usual manner. The incubation time was decreased to 15 minutes to reduce the possibility of heat inactivation. A few assays were made in 10 minutes and they agreed very well with the 15 minute assays. The entire denatured incubation mixture (2 ml.) was used to determine the α-ketobutyrate produced because the enzyme activity was very low at these dilutions.

In order to show that the activity of the inactivated enzyme had decreased to the level observed before it was adjusted to 0.1 M potassium phosphate, other portions of the inactivitated enzyme solution were adjusted at later periods. As shown in Table 9, up to 98% of the original activity could be restored by the immediate adjustment of the potassium phosphate concentration. Moreover, if the adjustment was made an hour later 88% of the activity

could be restored. Therefore, the addition of potassium phosphate does reverse the inactivation resulting from a low ionic environment. However, there appears to be a second, slower, inactivation which is irreversible.

Table 9. The reversal of the inactivation of the enzyme resulting from a low ionic environment by the addition of potassium phosphate[a]

KHP[b] conc.	% Activity		KHP addition hrs.
	Before[c]	After[d]	
1×10^{-1} M	100	---	----
2.5×10^{-5} M	60	96	0.00
		77	2.00
1×10^{-5} M	49 - 59	96	0.00
		91	0.25
		88	1.00
		77	2.00
5×10^{-6} M	39 - 49	98	0.00
		78	2.00

[a]The enzyme was assayed for 15 minutes in the usual manner. The entire denatured reaction mixture (2 ml.) was used for the determination of α-ketobutyrate.

[b]KHP: potassium phosphate.

[c]Before the addition of potassium phosphate.

[d]After the addition of potassium phosphate.

c. Effect of salts on the activity of enzyme. A different approach was used in determining the activity of the enzyme in various salts from the one used by previous workers (32,40,41). They prepared the enzyme in either 0.1 M sodium phosphate buffer, pH 7.2 or tris(hydroxymethyl)aminomethane buffer, pH 8.6 and used these solutions as their basis of comparison. Therefore, their solutions contained additional ionic species (buffer) which may have affected their results.

The present method (dilution) decreases the concentration of the additional ionic species to a greater extent; the concentration of the additional species is 1/400 of the added salt concentration. Salt solutions, 1×10^{-2} M, were prepared and adjusted to pH 7.2. The solutionw were assayed for 15 minutes in the usual manner. Again, the entire denatured enzyme reaction mixture was used for the determination the α-ketobutyrate produced. Table 10 shows the relative activity of the enzyme in various salt concentrations. These results agree very well with those obtained by other workers (32,40,41).

The results of the desalting and dilution experiments reveal that the enzyme is rapidly inactivated as a result of its presence in a low ionic environment. Dialysis against distilled water indicate that a total loss of enzyme activity occurs in the complete absence of salts.

Table 10. The activity of the enzyme in various salt concentrations[a]

Salt[b]	% Activity
Potassium phosphate	100
Potassium chloride	99
Sodium phosphate	79
Sodium chloride	77
Ammonium chloride	87
Lithium chloride	80

[a]The enzyme was assayed for 15 minutes in the usual manner. The entire denatured reaction mixture (2 ml.) was used for the determination of α-ketobutyrate.

[b]The salt concentration was 1×10^{-2} M.

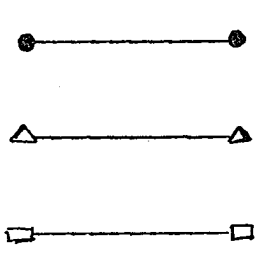
At 5×10^{-6} M potassium phosphate only 39% - 49% of the original activity is present. The immediate addition of potassium phosphate to a concentration of 0.1 M restores the activity. The magnitude of this restoration by potassium phosphate depends on the length of time the enzyme remains in a low ionic environment, since a second, slower, irreversible inactivation also takes place. Previous workers have shown that the salt effects depend on the monovalent cation and is not an ionic strength effect (32,40,41). The present work is in agreement with this interpretation.

F. Inactivation by L-Serine

The inactivation of the L-threonine dehydrase of sheep liver by L-serine is shown in Fig. 14 (32,40,41). Under the regular assay conditions of high substrate concentration, the production of α-ketobutyrate is linear with time, up to two hours. After this time, the production begins to decrease with time as a result of substrate depletion. However, under the same conditions, using 8-10 times the concentration of enzyme, the production of pyruvate from L-serine is not linear with time. Instead at pH 8.9, there is an initial burst of activity for about 2 minutes and then the activity decreases to a much slower rate which is linear up to two hours. Moreover at pH 7.2, the initial burst of activity lasts about 4 minutes and then the production of pyruvate decreases almost to zero.

Once the enzyme has been inactivated by L-serine, neither substrate appears to have an effect on the resulting enzyme activity. This is supported by the following experimental evidence. When L-threonine and L-serine are incubated together with the enzyme, results very similar to those obtained with L-serine alone are observed (32). When L-threonine is incubated with the enzyme for 30 minutes and L-serine added after this time, the production of α-keto acid decreases to a rate similar to that obtained with L-serine alone (40). Moreover, when the enzyme has

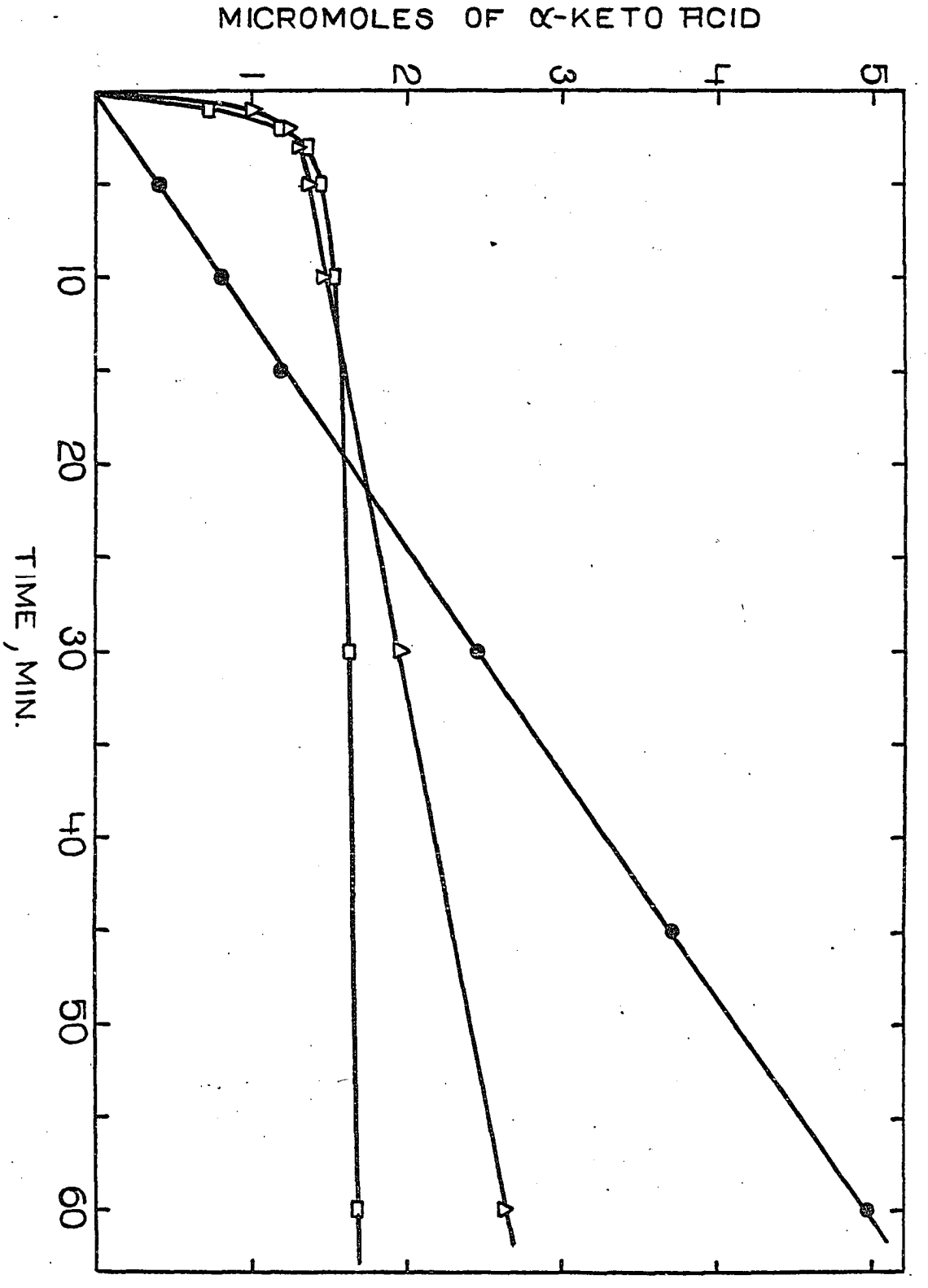
Fig. 14. ∝-Keto acid production as a function of time for the original enzyme.



Substrate: L-threonine, pH 8.9
5 Enzyme units

Substrate: L-serine, pH 8.9
40 Enzyme units

Substrate: L-serine, pH 7.2
40 Enzyme units


MICROMOLES OF α-KETO ACID
1
2
3
4
5
TIME, MIN.
10
20
30
40
50
60

been inactivated by L-serine, addition of L-threonine does not increase the rate of α-keto acid production (40).

However, the pH of the reaction mixture does affect the magnitude of this inactivation. For, if the enzyme is inactivated at pH 7.2 for 30 minutes, an increase in the pH, after this time, results in an increase in the rate of production of pyruvate. Thus L-serine modifies or alters the enzyme to a less active form. This alteration does not appear to be affected by the substrate but its magnitude is affected by the pH of the reaction mixture.

1. Preparation of altered enzyme

In order to learn more about the nature of this modification, a study of the properties of the altered form of the enzyme was undertaken. A Sephadex G-25, coarse, column, 2.5 x 62 cm. was prepared using 0.1 M phosphate buffer, pH 7.2. Enzyme prepared through the chromatography on Sephadex G-25, was used for these studies. Usually 3.3 ml. of enzyme solution, containing 200 to 1600 units per ml., were incubated with 1.1 ml. of 0.1 M phosphate buffer, pH 7.2 and 1.1 ml. of 0.1 M buffered L-serine, pH 7.2 for 15 minutes at 37°C. The ratio of the reactants is approximately the same as that used in the regular assay. After incubation, this mixture was added to the Sephadex G-25 column. The altered enzyme was eluted with 0.1 M phosphate buffer, pH 7.2. Four ml. fractions were collected. The

separation of the altered enzyme from L-serine and pyruvate was complete in 15 minutes. Ninhydrin solution was used to detect the presence of amino acids. Ninhydrin solution produced a faint blue color in those tubes containing the altered enzyme; however, this was equivalent to the color produced in the original enzyme solution by Ninhydrin solution. A typical elution pattern is shown in Fig. 15. The protein peak was combined for further studies. Although the altered enzyme was stable at 2°C for at least 5 days, fresh altered enzyme solution was prepared each day. The time of incubation of the original enzyme and L-serine had very little effect on the resulting activity of the altered enzyme.

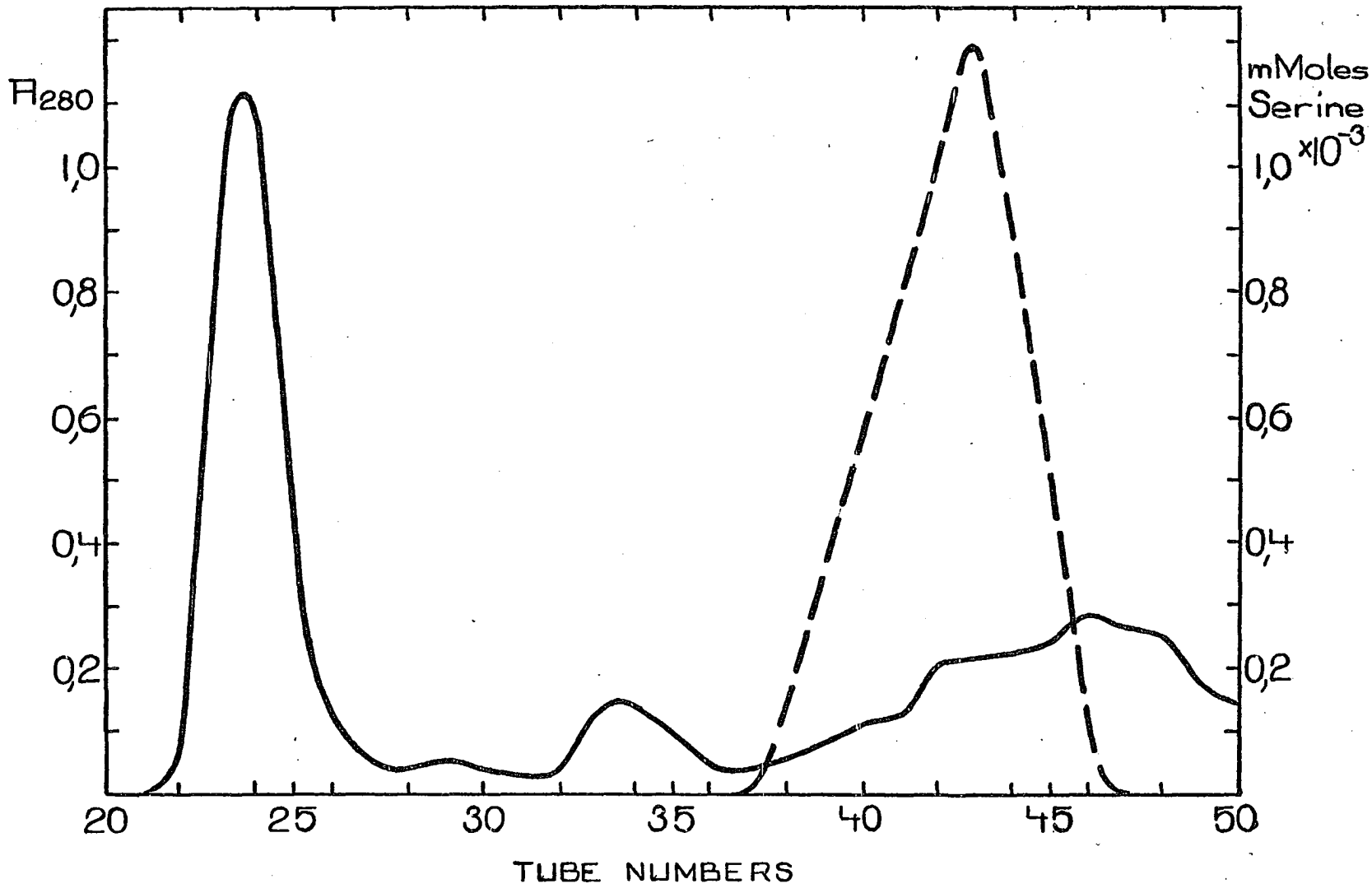
2. Activity of altered enzyme

As a control, an enzyme solution was incubated with L-threonine for 15 minutes and subjected to chromatography on Sephadex G-25. The enzyme solution, before and after the above treatment was assayed in the following manner. A mixture of 0.8 ml. of enzyme solution, 0.1 ml. of 0.1 M phosphate buffer, pH 7.2 and 0.3 ml. of 0.5 M tris-carbonate buffer, pH 8.95 was incubated at 37°C for 5 minutes. Then 0.3 ml. of 0.1 M buffered substrate, pH 8.9 was added to this mixture and incubated for 30 minutes. The reaction was stopped by the addition of 0.5 ml. of 25% trichloroacetic acid. After centrifugation, one ml. was used to determine

Fig. 15. The elution pattern of the chromatography of L-threonine dehydrase, inactivated by L-serine, on Sephadex G-25.

———— A_{280}

— — — Millimoles of serine


A280
1,0
0,8
0,6
0,4
0,2
mMoles
Serine
1,0 x10-3
0,8
0,6
0,4
0,2
20
25
30
35
40
45
50
TUBE NUMBERS

the α-keto acid produced. As shown in Table 11, the passage of the enzyme through Sephadex G-25 does not affect the purity index of the enzyme. The decrease in the protein concentration and units after chromatography is due to dilution resulting from the chromatography.

Table 11. The activity of the enzyme after incubation with L-threonine for 15 minutes

Enzyme	Protein mgm.	Units[a]	Purity index
Before chromatography	6.53	90.0	14.3
After chromatography	1.01	14.2	14.1
After chromatography + pyridoxal phosphate	1.01	15.7	15.5

[a]Micromoles of α-ketobutyrate produced per hour at 37°C, pH 8.9.

The altered enzyme was assayed in the above manner. The term altered enzyme refers to that enzyme solution which has been incubated with 0.1 M buffered L-serine, pH 7.2 for at least 15 minutes and isolated by chromatography on Sephadex G-25. An equal quantity of the original enzyme, based on the protein concentration was also assayed in the above manner and compared to the altered enzyme. As shown in Table 12, the altered enzyme produced much less

α-ketobutyrate and pyruvate than the original enzyme in 30 minutes.

Table 12. The activity of the enzyme after incubation with L-serine for 15 minutes

Enzyme	Protein mgm.	α-Ketobutyrate[a]	Pyruvate[a]
Original	2.29	52.00	5.85
Original +PLP[b]	2.29	61.00	6.60
Altered	2.29	11.37	1.72
Altered +PLP[b]	2.29	11.44	1.81

[a]Micromoles of product produced in 30 minutes at 37°C, pH 8.9.

[b]PLP: pyridoxal phosphate.

The effect of pyridoxal phosphate on the activity of the altered and original enzyme is also shown in Tables 11 and 12. The assay procedure differed from the above procedure in that 0.1 ml. of pyridoxal phosphate (100 micromoles) was substituted for the 0.1 ml. of phosphate buffer. Before the assay was started, 0.8 ml. of the enzyme solution (altered or original) was incubated with the 0.1 ml of pyridoxal phosphate for one hour. Afterwards, 0.3 ml. of tris-carbonate buffer, pH 8.95 was added and the remainder

of the procedure was the same as above. The pyridoxal phosphate in the reaction mixture was 6.67×10^{-5} M and it had very little effect on the activity of the original or altered enzyme. Even when the pyridoxal phosphate concentration was varied from 2×10^{-4} to 3×10^{-7} M, no significant increase in activity was observed. Since pyridoxal phosphate reacts with 2,4-dinitrophenylhydrazine, unusually high blanks were obtained. Therefore, all of the samples were read against a denatured enzyme blank, containg no pyridoxal phosphate. Corrections were made for the presence of pyridoxal phosphate. At 6.67×10^{-5} M pyridoxal phosphate the blank has an optical density between 0.090 and 0.120. Blanks for pyridoxal phosphate concentrations above 2×10^{-4} M were too dark to give reliable results.

3. Effect of substrate on the activity of altered enzyme

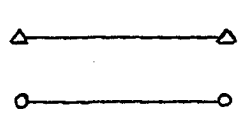
A mixture of 6.0 ml. of altered enzyme, 0.75 ml. of 0.1 M phosphate buffer, pH 7.2 and 2.25 ml. of 0.5 M tris-carbonate buffer, pH 8.95 was prepared and incubated at 37°C for 5 minutes. Then 2.25 ml. of 0.1 M buffered substrate, pH 8.9 was added. An aliquot, consisting of 1.2 ml. of the reaction mixture, was removed periodically and added to a test tube containing 0.4 ml. of 25% trichloroacetic acid. After centrifugation, one ml. of this solution was used to determine the production of α-keto acid.

The above reaction mixture was at pH 8.9. For reaction mixtures at pH 7.2, 2.25 ml. of 0.1 M phosphate buffer, pH 7.2 and 2.25 ml. of 0.1 M buffered substrate, pH 7.2 were substituted into the above procedure. When pyridoxal phosphate was included in the reaction mixture, it replaced the 0.75 ml. of phosphate buffer. Again the altered enzyme was incubated with the pyridoxal phosphate for one hour, before the tris-carbonate buffer was added. The same altered enzyme solution was used for all the measurements.

When L-threonine was used as the substrate, aliquots were removed from the reaction mixture 5, 10, 15, 30, 45, and 60 minutes after the addition of the substrate. The results, as shown in Figs. 16 and 17, reveal that the production of α-ketobutyrate at pH 7.2 or 8.9 is not linear with time; instead, the rate of production increases with time.

When L-serine was used as the substrate, aliquots were removed from the reaction mixture 1, 3, 5, 10, 20, 30, and 60 minutes after the addition of the substrate. The results, as shown in Fig. 18, reveal that the production of pyruvate at pH 7.2 or 8.9 is not linear with time. Instead, the shape of the curves is the same as the shape for the action of L-serine towards the original enzyme, shown in Fig. 14.

Fig. 16. α-Ketobutyrate production at pH 8.9 as a function of time for the altered enzyme.



− Pyridoxal phosphate

+ Pyridoxal phosphate

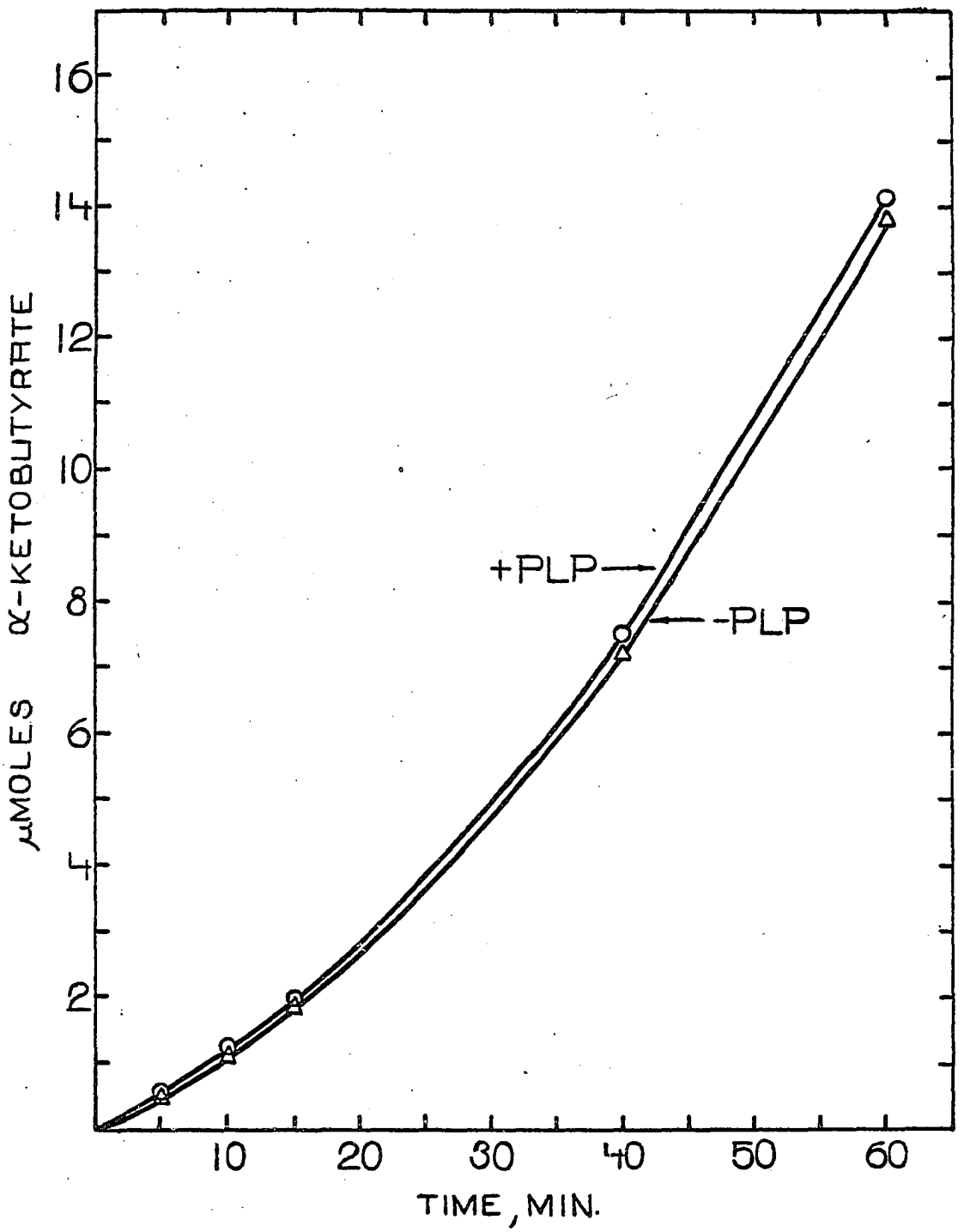
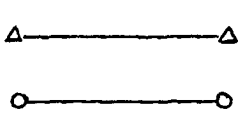

16
14
12
10
8
6
4
2
μMOLES α-KETOBUTYRATE
+PLP
-PLP
10
20
30
40
50
60
TIME, MIN.

Fig. 17. α-Ketobutyrate production at pH 7.2 as a function of time for the altered enzyme.



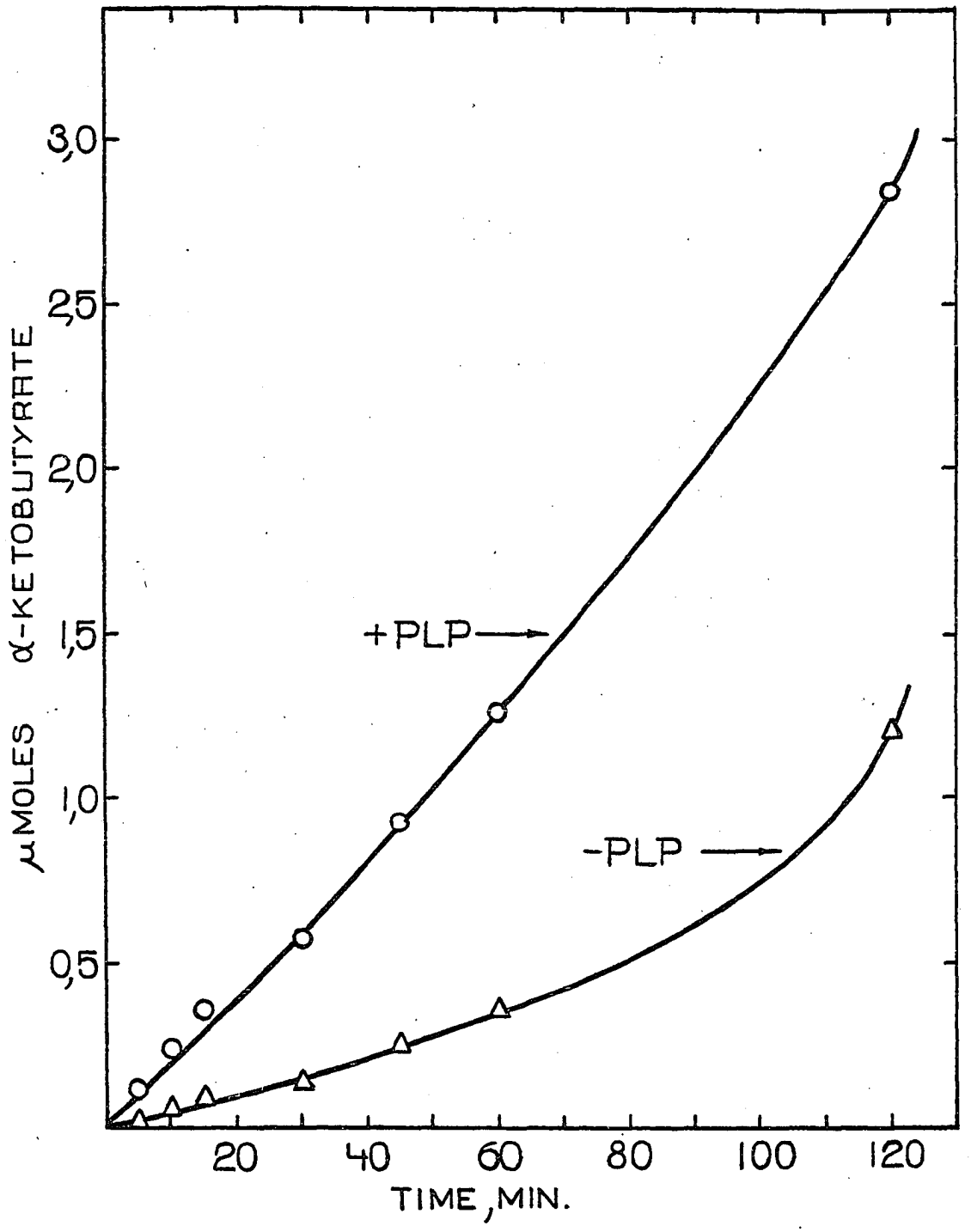
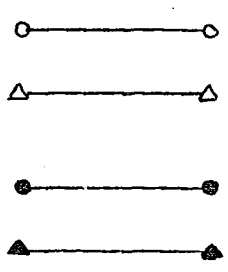

3,0
2,5
2,0
1,5
1,0
0,5
μMOLES α-KETOBUTYRATE
+PLP
-PLP
20
40
60
80
100
120
TIME, MIN.

Fig. 18. Pyruvate production as a function of time for the altered enzyme.

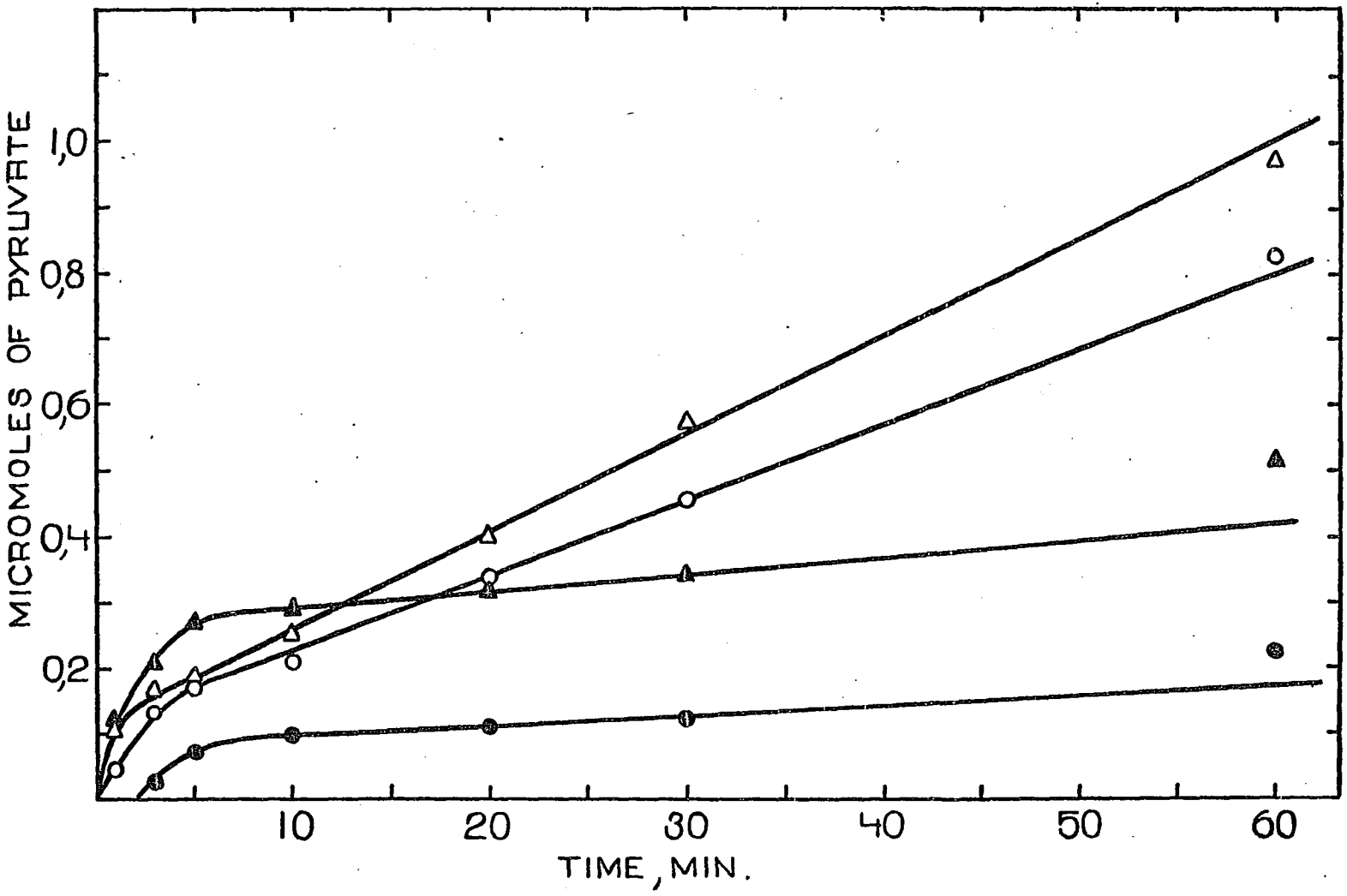


− Pyridoxal phosphate, pH 8.9

+ Pyridoxal phosphate, pH 8.9

− Pyridoxal phosphate, pH 7.2

+ Pyridoxal phosphate, pH 7.2


MICROMOLES OF PYRUVATE
1,0
0,8
0,6
0,4
0,2
10
20
30
40
50
60
TIME, MIN.

4. Effect of pyridoxal phosphate on the activity of altered enzyme

Nishimura and Greenberg observed that when the enzyme is inactivated by L-serine, the coenzyme appeared to be resolved (32). Dialysis of the inactivated enzyme, followed by the addition of pyridoxal phosphate restored 95% of the enzyme activity. Therefore, it appeared that the modification by L-serine facilitated the dissociation of coenzyme from apoenzyme. Since dialysis only removed pyruvate, serine and the resolved coenzyme from the enzyme solution and probably did not interact with the altered enzyme, chromatography on Sephadex G-25 should yield similar results. Figs. 16, 17, and 18 show the effect of pyridoxal phosphate on the altered enzyme. It is interesting that pyridoxal phosphate does not alter the shape of the curves. Although pyridoxal phosphate does increase the production of α-keto acid in each case, the increase is small, especially at pH 8.9. At pH 7.2, this increase is more significant; the α-keto acid production doubles. It should be pointed out that the resolution studies of Nishimura and Greenberg were done at pH 7.2. We have been unable to restore the altered enzyme to 95% of its original activity by the addition of pyridoxal phosphate, at pH 7.2. However, we have been able to increase the activity of the altered enzyme from 3% to 7% of the original by the addition of pyridoxal

phosphate, at this pH. Although this effect is small, it is significant. The effect of pyridoxal phosphate on the altered enzyme, at pH 7.2 was observed only recently and should be studied in more detail.

In order to allow time for the resolution of the coenzyme by L-serine, the altered enzyme was stored for 24 hours, at 2°C. However, assays of this solution yielded results similiar to those above. Therefore, L-serine does not appear to resolve the coenzyme under the above conditions.

5. Reactivation of altered enzyme by L-threonine

The slope of the product vs time curves in Figs. 16, 17, and 18 is equal to the average velocity of the reaction. When this curve is linear, the average velocity is equal to the instantaneous velocity at any point on the curve. However, when the curve is not linear, in order to determine the instantaneous velocity, it is necessary to obtain the slope of the tangent to the curve, at that particular point. The instantaneous velocity of the altered enzyme towards L-threonine was determined from tangents to the curve. As the reaction time increased, the velocity of the altered enzyme approached the velocity of the original enzyme. Therefore, it appears that L-threonine reverses the inactivation of the enzyme caused by L-serine.

In order to show that a reversal of the inactivation by L-serine does occur when L-threonine is incubated d

with the altered enzyme, the following experiment was performed. The altered enzyme was prepared in the usual manner. A mixture of 6.0 ml. of altered enzyme solution, 2.0 ml. of 0.5 M buffered L-threonine, pH 8.9 and 2.0 ml. of tris-carbonate buffer, pH 8.95 was incubated at 37°C for 10, 60, 105, or 150 minutes. After incubation, the mixture was added to the Sephadex G-25, coarse, column and eluted with 0.1 M phosphate buffer, pH 7.2 in 15 minutes. Four ml. fractions were collected. The resultant enzyme solution was assayed following the same procedure used to determine the activity of the altered enzyme. Table 13 shows the activity of the original, altered and reactivated enzyme.

Table 13 clearly illustrates that the activity of the altered enzyme is restored by its incubation with L-threonine. Incubation of the altered enzyme with L-threonine for 105 minutes, restores 88% of the original activity. Longer incubations do not increase the reactivation. The apparent higher restoration of the enzyme activity towards L-serine is believed to result from the departure from linearity of the production of pyruvate with time.

As shown in Fig. 19, reactivated enzyme, prepared by the incubation of altered enzyme and L-threonine for 90 minutes, produced α-ketobutyrate linearly, after a short lag period. However, when the incubation time was increased to 120 minutes, no lag period was observed.

Table 13. Reactivation of altered enzyme by incubation with L-threonine

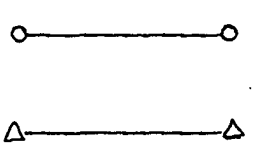
Enzyme	Incubation[a] time	Substrate	Protein mgm.	Product[b]	Product / Protein	% Activity
Original	0	Threonine	14.75	150	10.10	100
Altered	0	Threonine	3.08	5.22	1.68	17
Altered	10	Threonine	0.80	1.78	2.12	22
Altered	60	Threonine	0.81	4.23	5.20	52
Altered	150	Threonine	0.75	6.30	8.40	83
Original[c]	0	Threonine	14.75	170	11.50	100
Altered[c]	0	Threonine	3.08	6.40	2.07	18
Altered[c]	10	Threonine	0.80	2.21	2.76	24
Altered[c]	60	Threonine	0.81	5.00	6.17	55
Altered[c]	150	Threonine	0.75	6.92	9.28	81
Original	0	Serine	15.00	14.20	0.95	100
Altered	0	Serine	3.08	0.87	0.28	30
Altered	10	Serine	0.80	0.27	0.32	34
Altered	150	Serine	0.75	0.72	0.97	102
Original[c]	0	Serine	15.00	17.50	1.17	100
Altered[c]	0	Serine	3.08	1.00	0.32	26
Altered[c]	10	Serine	0.80	0.30	0.37	32
Altered[c]	150	Serine	0.75	0.88	1.25	107

[a]Duration of incubation of altered enzyme with L-threonine.

[b]The micromoles of α-keto acid produced in 30 minutes at pH 8.9, 37°C.

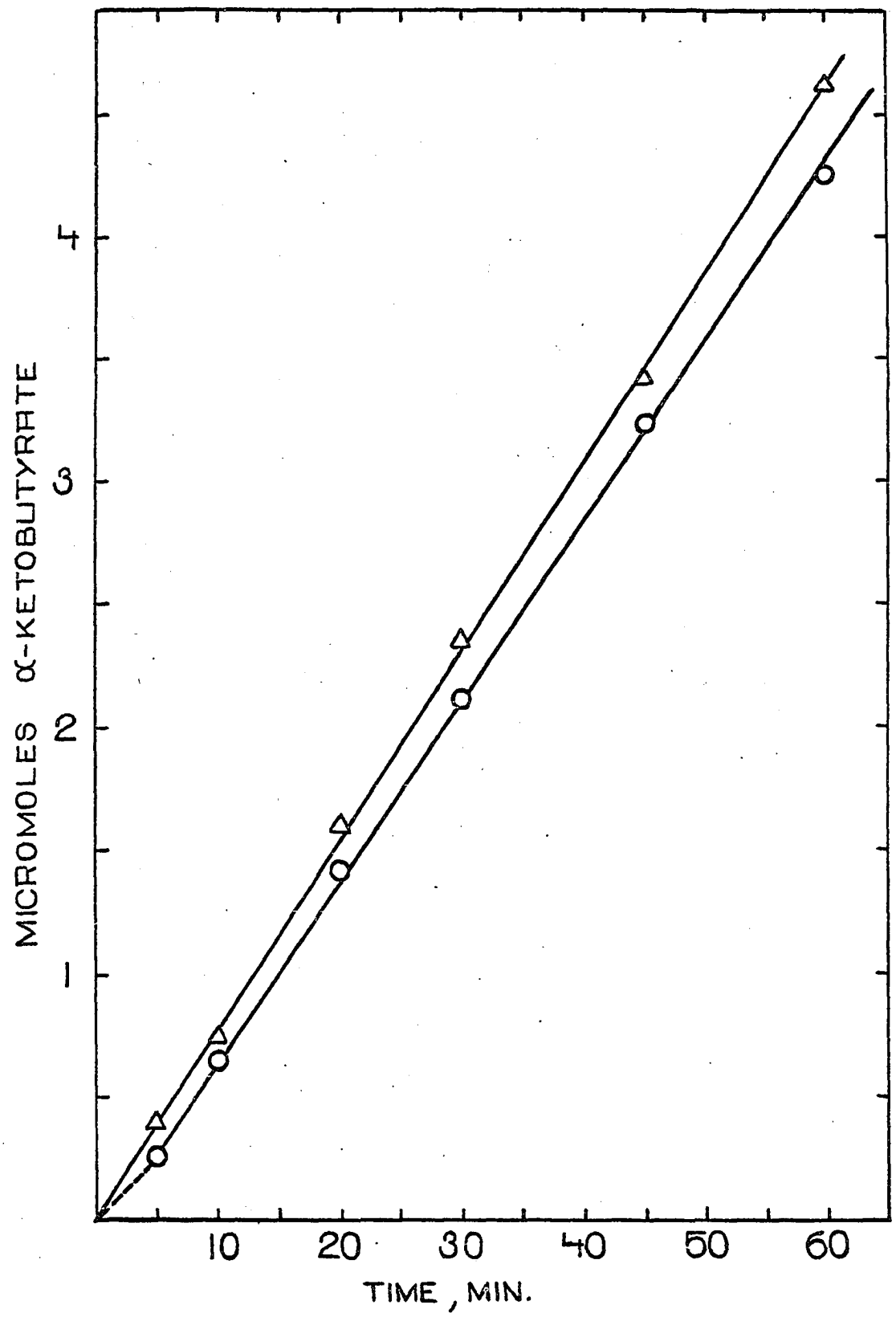
[c]Samples contained pyridoxal phosphate.

Fig. 19. α-Ketobutyrate production at pH 8.9 as a function of time for the reactivated enzyme.



90 minute preincubation with L-threonine

120 minute preincubation with L-threonine


MICROMOLES α-KETOBUTYRATE
1
2
3
4
10
20
30
40
50
60
TIME, MIN.

6. K_M of the altered enzyme

The same assumptions made in determining K_M, the Michaelis constant, for the original enzyme were used to determine K_M for the altered enzyme (32,40,41). It was assumed that the enzyme reacts with the substrate to form a Michaelis complex, which can either dissociate to free enzyme and substrate or decompose irreversibly into free enzyme and product, as shown below. Lineweaver and Burke developed a relationship based on these assumptions in which when $\frac{1}{v}$ is plotted against $\frac{1}{s}$ the slope equals $\frac{K_M}{V_M}$ and the y intercept equals $\frac{1}{V_M}$ (89). K_M is obtained by dividing the slope by the intercept.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

$$\frac{1}{v} = \frac{K_M}{V_M}\frac{1}{s} + \frac{1}{V_M}$$

E = enzyme

s = substrate

ES = Michaelis complex (enzyme-substrate complex)

P = product

v = initial velocity of one unit of enzyme

K_M = Michaelis constant

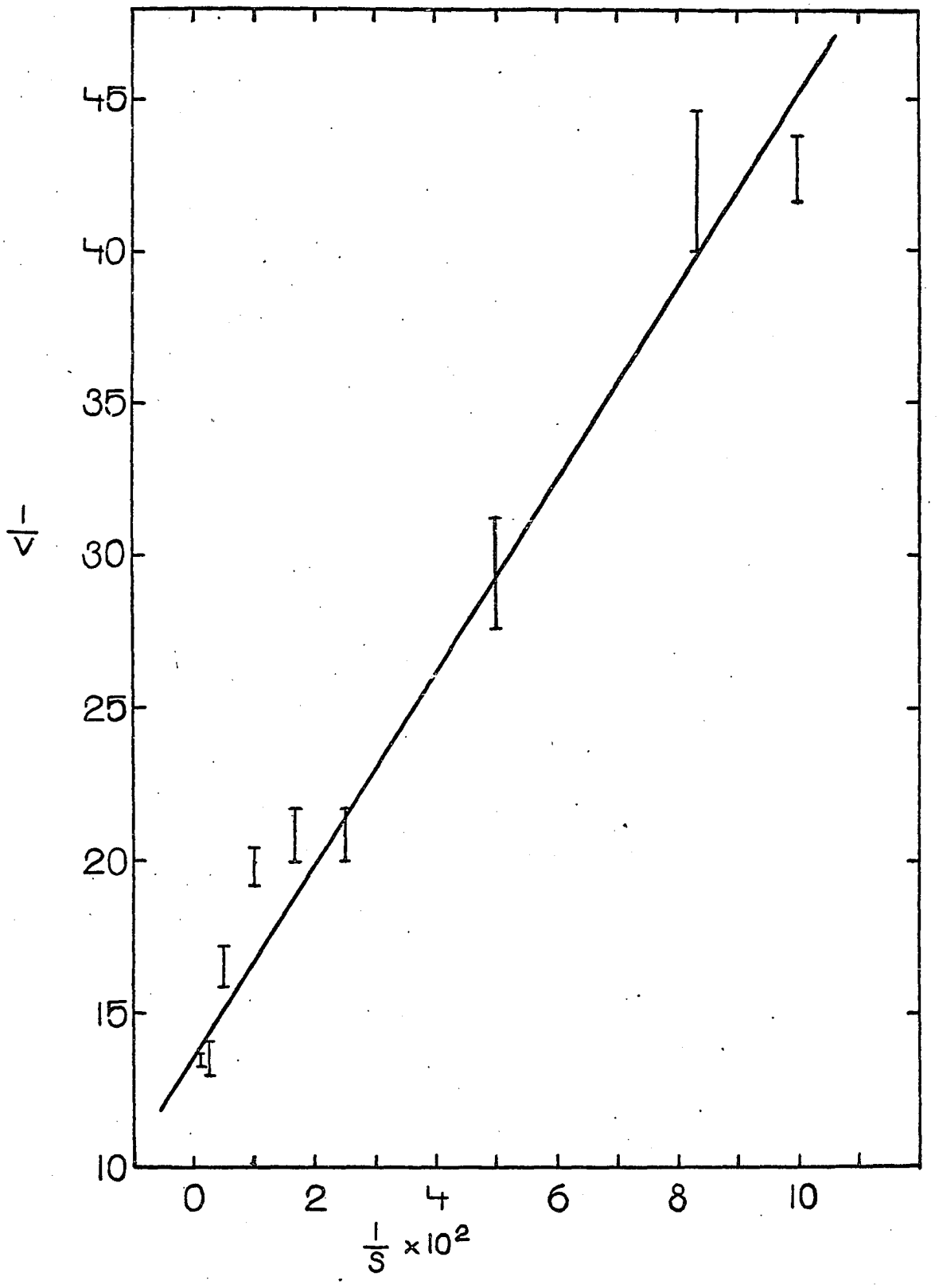
V_M = maximum velocity

The production of α-ketobutyrate is linear during the first 10 minutes of the reaction of the altered enzyme on L-threonine, as shown in Fig. 16. Therefore, the altered

enzyme was assayed for 10 minutes in the regular manner, using various concentrations of buffered L-threonine, pH 8.9. The velocities were converted to the base of one unit of enzyme as defined in the analytical methods.

A plot of $\frac{1}{v}$ vs $\frac{1}{s}$ for the altered enzyme is shown in Fig. 20. By drawing the most extreme points possible through the data, a variation of 3×10^{-4} M in the K_M was observed. A K_M of 2.48×10^{-3} M was determined for the altered enzyme, at pH 8.9. This value agrees very closely with the K_M for the original enzyme, 2.54×10^{-3} M, at pH 8.9. Assuming that K_M equals K_S, the dissociation constant of the Michaelis complex, then the affinity of both enzymes is the same. If this is true, L-serine does not affect the rate of the formation of the Michaelis complex. Since L-serine inactivates the enzyme, V_M, as expected, is much lower in the case of the altered enzyme.

Fig. 20. Reciprocal plot of initial velocity, v, versus substrate concentration, s, of the altered enzyme for L-threonine at pH 8.9.


45
40
35
30
25
20
15
10
1/V
0
2
4
6
8
10
1/S x10²

V. DISCUSSION

Any serious study of an enzyme requires that it be in a highly purified form. The present preparations of L-threonine dehydrase of sheep liver do not meet this requirement. However, a purity index of 1600 units per mgm. protein has been achieved by the present purification procedure. Although this value is higher than any reported in the literature, comparisons are complicated by differences in the assay procedure. Our reaction was performed at pH 8.9, whereas Nishimura and Greenberg's, who reported an activity of 773 units per mgm. protein, reaction was performed at pH 7.2. (32). The enzyme is more active at pH 8.9; therefore, it is possible that a value of 773 at pH 7.2 is equivalent to 1600 at pH 8.9. It should be pointed out that the initial concentration of enzyme in our sheep liver homogenates was less than 1/2 those reported by Nishimura and Greenberg.

In order to get a better idea of the purity of our enzyme preparations, the results of the experiments on the sedimentation and electrophoretic mobility are considered. The presence of three peaks in the schlieren pattern and four or more bands on the polyacrylamide gel columns indicate that these preparations contain at least three proteins. The calculated sedimentation constants, although approximate,

suggest that the components differ considerably in size, as shown by the molecular weights derived from these sedimentation constants. These molecular weights are valid only if the assumptions made are valid; therefore, they should be regarded only as approximate molecular weights.

When these preparations were placed on Sephadex G-200, which separates molecules up to a molecular weight of 150,000 to 200,000, no apparent separation was observed. This is consistent with the molecular weight of the two larger proteins. The small protein can be explained, if it is not spherical. An asymmetric protein would resemble a larger protein in its action towards Sephadex.

Since schlieren patterns of preparations with a purity index above 500 units per mgm. protein could not be obtained, it is impossible to assess the purity of these preparations. However, on the basis of the experiments on ultracentrifugation and disc gel electrophoresis one more purification step may yield a homogeneous preparation of the enzyme. Sucrose gradient centrifugation or preparative polyacrylamide gel electrophoresis are excellent possibilities, since they are preparative applications of the above analytical methods. Other possible methods include chromatography on columns of hydroxy apartite, agar or Bio-Gel (capable of separating substances up to a molecular weight of 300,00; obtained from Calbiochem; Los Angeles, Calif.).

Improvements on the present purification procedure also should be considered. If one could control the concentration of enzyme in the sheep liver, this would help tremendously. Various workers have induced the dehydrases of E. coli by adding high concentrations of DL-threonine, DL-serine, L-leucine or glycine to their diet (90,91,92). Greenberg et al. have induced the threonine dehydrase of rat liver by adding DL-threonine intraperitoneally (28,93). In addition, Potter and Ono have shown that the diet affects the threonine dehydrase activity of rat livers (94).

Surprisingly, the two most active enzyme preparations were not from sheep livers with unusually high concentrations of enzyme. Instead, an unusually high purification resulted from the second ammonium sulfate precipitation. Attempts made to determine the cause of this unusual increase in purification, by varying the experimental techniques involved in this step, were unsuccessful. It is possible that the previous heat treatment removed an agent from the enzyme solution, thus accounting for this increase.

Chromatography on DEAE-Sephadex is very rewarding, since it gives a 5-10 fold purification and also two active fractions. However, 30% of the enzyme could not be removed from the column, even when the phosphate buffer concentration was increased to 1.0 M. Although the present conditions for chromatography give satisfactory results,

improvements are needed.

The two fractions obtained from the chromatography on DEAE-Sephadex are very similar in their properties, i.e. specificity, spectrum, and electrophoretic mobility. The only differences observed were their elution pattern and purity index. Therefore, it is possible that a portion of the enzyme is in the form of a complex with contaminating material, thus accounting for fraction II. However, the possibility that the fractions are isozymes can not be excluded. Isozymes are enzymes from the same source which catalyze the same reaction but have different physical properties.

It is not surprising that the addition of α-keto acids did not activate the enzyme. Otherwise, one would expect an autocatalytic process to take place during the normal reaction. The inhibition caused by the α-keto acids at 1×10^{-3} M is probably a result of product inhibition. It is interesting that no aminotransferase has been isolated with significant activity towards L-threonine. In addition, L-threonine is transaminated very slowly in model systems containing pyridoxal phosphate (95,96). Therefore threonine dehydrase may play a major role in the deamination of threonine in biological systems.

All of the dehydrases studied thus far are activated by monovalent cations, especially K^+ and NH_4^+ (1,30,32,37-41).

In addition, tryptophanase and the B-protein of tryptophan synthetase, both of which have serine dehydrase activity, are also activated by K^+ and NH_4^+ (38,39,97). Although Na^+ inhibited many of these enzymes, it activates our enzyme slightly (40). Holzer observed that NH_4^+ had the greatest effect on the yeast threonine dehydrase (37). This is interesting since ammonia is one of the products of the reaction. As expected, Holzer observed an autocatalytic process during the normal reaction (98). In the case of our enzyme K^+ has the greatest effect and no autocatalytic process involving NH_4^+ has been observed.

Happold and Beechey postulated four methods of activation by K^+ and NH_4^+ and discussed the consequences of each (99). The four methods are:

1. That K^+ or NH_4^+ are essential for activity.

2. That K^+ or NH_4^+ are engaged in anchoring the substrate or coenzyme to an active centre.

3. That K^+ or NH_4^+ affect the organization of the enzyme molecule.

4. That K^+ or NH_4^+ accelerate the rate of breakdown of the enzyme-substrate complex.

According to the discussion, if methods 1 or 2 are involved, an increase in K^+ concentration would result in an increase in the affinity of enzyme for substrate, thus lowering the K_M. The maximum velocity would remain

unchanged. In contrast, if methods 3 or 4 are involved, an increase in K^+ would result in an increase in V_M and K_M would remain unchanged. Davis and Metzler have determined the effect of K^+ on the K_M and V_M of the sheep liver L-threonine dehydrase (40,41). At pH 8.6, in the presence of 0.002 M Potassium chloride, the Michaelis constant was 8.3×10^{-4} M compared to 1.9×10^{-3} M in the absence of added potassium chloride. The maximum velocity was unchanged. This data is consistent with methods 1 or 2. Our results on the initial inactivation of the enzyme of the enzyme in a low ionic environment and its subsequent reactivation does not enable us to discriminate between the postulated methods of activation. However, the fact that there is also a slow irreversible inactivation following the initial inactivation is not consistent with methods 2 and 4. Therefore, method 1 is implicated but the present data is not sufficient to prove any of the possibilities mentioned.

The inability of the desalting, dilution or dialysis experiments to resolve the coenzyme indicates the tenacity in which it is bound to the apoenzyme. Although the apparent resolution of the coenzyme has been observed using L-serine, this interpretation is not consistent with the present data. The principle observations which contradict the hypothesis of resolution are given below:

1. It is impossible to restore the activity of the

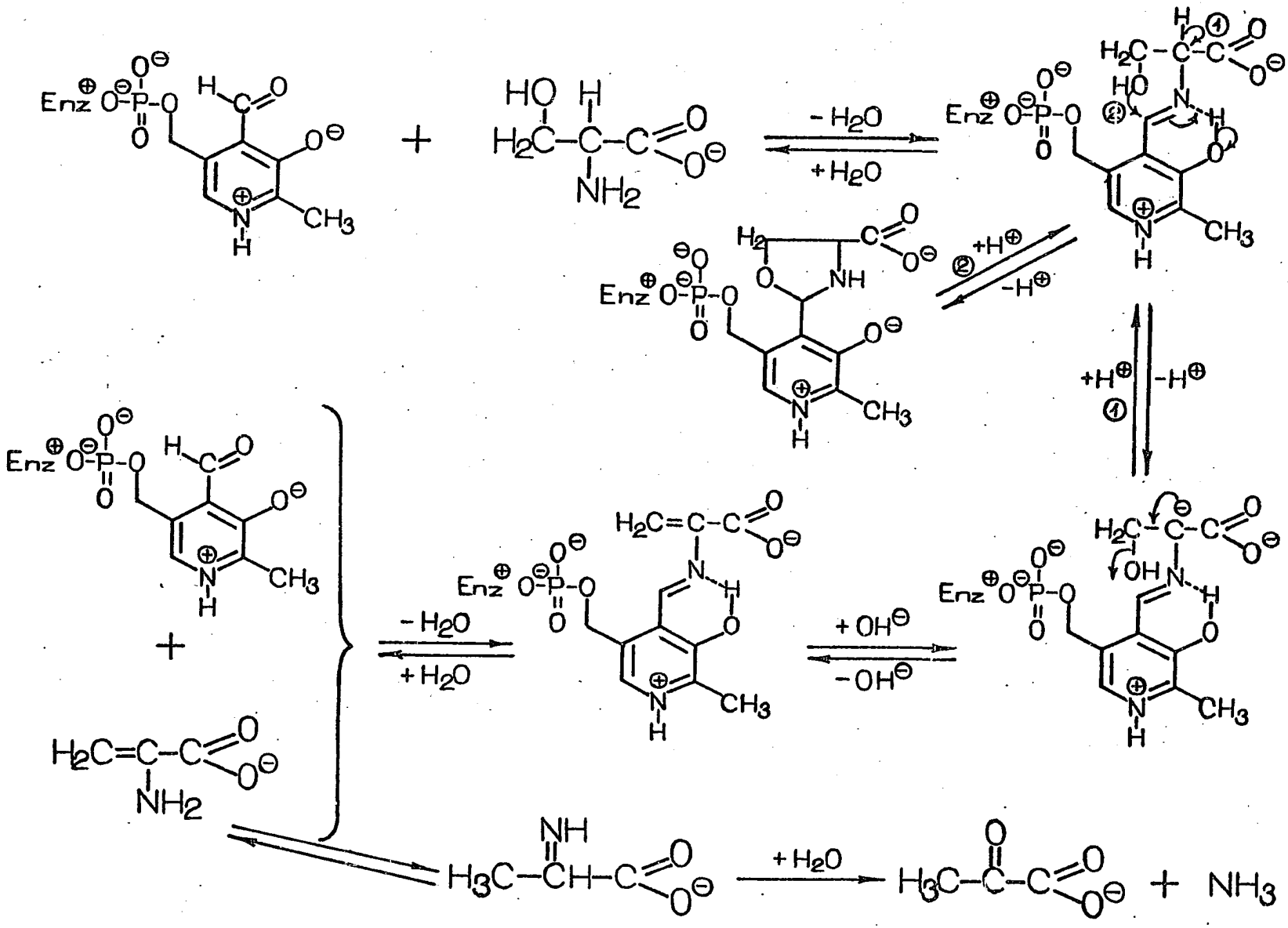
altered enzyme by its incubation with added pyridoxal phosphate. Although a small increase in activity is observed when pyridoxal phosphate was added at pH 7.2, prolonged standing did not affect the increase by pyridoxal phosphate.

2. The possibility that the apoenzyme is unstable is eliminated since the addition of L-threonine restores the activity under the same conditions.

3. The reactivation by L-threonine is not consistent with a mechanism in which the coenzyme has been removed.

Therefore, a new mechanism is proposed for the inactivation of the enzyme by L-serine, as shown in Fig. 21. In this mechanism L-serine forms an aldimine with the carbonyl carbon of pyridoxal phosphate. Free rotation around the β-carbon of serine allows the hydroxylic oxygen to come in close proximity to the aldimine carbon. A nucleophilic attack by the oxygen on the aldimine carbon would result in the formation of an oxazolidine ring as shown in reaction 2 of Fig. 21. This altered complex would be in equilibrium with the normal ES complex. Therefore reactions 1 and 2 would be in competition for the normal ES complex. The formation of the oxazolidine ring would prevent the liberation of free enzyme. At high serine concentration all of the enzyme would normally be in the form of an enzyme-substrate complex; therefore most of the enzyme would be in the altered form.

Fig. 21. The proposed mechanism of the inactivation of L-threonine dehydrase by L-serine.


Enz
$-H_2O$
$+H_2O$
$+H^{\oplus}$
$-H^{\oplus}$
$+OH^{\ominus}$
$-OH^{\ominus}$
$+H_2O$
$+ NH_3$

Removal of the excess serine by chromatography on Sephadex G-25 would result in a solution containing both normal and altered ES complexes. Thus incubation with L-threonine would shift the equilibrium towards the normal enzyme-serine complex. This complex would dissociate to free enzyme and pyruvate. The free enzyme would then react with L-threonine. Thus at the end of 120 minutes about 88% of the enzyme is in the form of enzyme-threonine complex.

The altered enzyme solution's action towards L-serine is explained as follows. When the excess L-serine is removed, the equilibrium of the complexes shifts towards the normal enzyme-serine complex, which can dissociate to give free enzyme and serine. Therefore, when the enzyme is incubated with L-serine, an initial burst of pyruvate production occurs until the equilibrium has shifted towards the altered enzyme-serine complex.

The effect of pyridoxal phosphate on the enzyme is more difficult to explain. One possibility is that the carbonyl carbon of the coenzyme is normally in the form of an aldimine with an ϵ-amino group of lysine. Therefore, when an ES complex is formed this amino group is freed. In the case of the altered enzyme-serine complex, the free amino group would react with the added pyridoxal phosphate. This newly formed aldimine would then compete with the altered coenzyme for its site on the apoenzyme. If the rate of reactivation

by pyridoxal phosphate is much slower than the rate of reactivation by L-threonine at pH 8.9, the reactivation by pyridoxal phosphate would be masked at this pH. If the relative rates of reactivation are reversed at pH 7.2, reactivation by pyridoxal phosphate would be observed at this pH.

The oxazolidine ring involving pyridoxal phosphate and L-serine, has no direct support; however, analagous structures have been cited in the literature (8,11,20,100-103). The sulfur analog, cysteine, is known to form a similar thiazolidine ring with pyridoxal phosphate (8,20,100-102). Cori et al. have proposed a structure for the role of pyridoxal phosphate in phosphorylase involving a similar five-membered ring with group X in the place of the hydroxylic oxygen (11,100). They have postulated that X can be an oxygen, sulfur or nitrogen atom. Dempsey and Christensen have proposed a similar structure for the binding of pyridoxal phosphate to bovine plasma albumin (103). All of these structures absorb at 330 mμ. The observance of a peak at 330 mμ on the addition of L-serine to our enzyme would support the present proposal. In nonenzymatic systems, oxazolidine compounds can be prepared by reacting carbonyl compounds with β-amino alcohols (104,105). These oxazolidine compounds are usually in tautomeric equilibrium with the open chain Schiff bases.

Possible future experiments related to the inactivation of the enzyme by L-serine are given here. Similar experiments should be done using the analog of serine, cysteine. Hydroxylamine is reported to resolve the enzyme (32). It would be interesting to see if the results of resolution studies using hydroxylamine would fit the present proposal. There is no reason that the mechanism should necessarily be the same, especially since a similar structure would involve a three membered ring. However, the homolog, ethanolamine, would be interesting in this respect. Since pyridoxal phosphate does restore some activity at pH 7.2, the effect of analogs of pyridoxal phosphate should be observed.

The use of Sephadex has been very fruitful in the present work. Its molecular sieving properties has allowed the study of many properties of the enzyme by a different approach. Although some of these properties had been studied previously, the use of a different approach enabled us to corroborate the previous work; to attain new information concerning the properties of the enzyme; and to evaluate the interpretations related to these properties.

VI. SUMMARY

1. L-threonine dehydrase of sheep liver has been purified by a combination of heat treatments, ammonium sulfate fractionations and chromatography on DEAE-Sephadex. Purity indices between 300-500 units per mgm. protein usually are obtained, although in exceptional cases, purity indices as high as 1600 units per mgm. protein have been obtained.

2. Chromatography on DEAE-Sephadex yielded two fractions containing enzyme activity. Both fractions were active towards L-threonine and L-serine and neither was activated by pyridoxal phosphate, adenylic acid, adenosine diphosphate or glutathione. Fraction I, by order of elution, contained enzyme which had been purified 5-10 fold over that which had been placed on the column; fraction II contained enzyme purified 2 fold.

3. Ultracentrifugation studies indicated that at least three components are present in the enzyme solution after chromatography on DEAE-Sephadex. Approximate sedimentation constants were calculated to be 4.75-5.15, 8.60-8.80, and 11.50-12.00. The molecular weight of these components is approximately 60,000-70,000; 110,000-180,000; and 225,000-290,000 respectively. The heterogeneity of these preparations was also indicated by the presence of four or more

bands on disc gel electrophoresis columns.

4. A series of methods for concentrating the enzyme were investigated and precipitation by ammonium sulfate or vacuum filtration proved to be the best.

5. Fractions I and II absorb at 278 mμ and between 405 mμ - 415 mμ. The 278 mμ peak corresponds to the aromatic amino acids and the 405 mμ - 415 mμ peak is believed to be caused by bound pyridoxal phosphate.

6. The enzyme is stable at room temperature, at least 12 hours; refrigerated, at least one month; and frozen, at least 6 months.

7. The enzyme is inactive in the absence of an ionic environment. The immediate addition of potassium phosphate restores the activity. The amount of restoration by potassium phosphate depends on the time of its addition, for a slower irreversible inactivation also takes place. The enzyme is most active in the presence of K^+.

8. The enzyme acts on L-serine as a substrate but is rapidly inactivated in the process, partially at pH 8.9 and more completely at pH 7.2. However, incubation with L-threonine at pH 8.9 leads to a gradual restoration of activity up to 88% of the original. The addition of pyridoxal phosphate to the inactive enzyme has no effect upon the activity or rate of reactivation at pH 8.9. However, at pH 7.2, the addition of pyridoxal phosphate leads to a doubling of the

small activity towards L-threonine. These observations are discussed in terms of the proposed formation of an oxazolidine ring, between serine and the bound coenzyme.

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