Snoke, J. E. (1955), *J. Biol. Chem. 213*, 813.
Snoke, J. E., and Bloch, K. (1955), *J. Biol. Chem. 213*, 825.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Strumeyer, D. H. (1959), Doctoral Dissertation, Harvard University, Cambridge, Mass.
Strumeyer, D., and Bloch, K. (1962), *Biochemical Prepn. 9*, 52.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.

The Mechanism of the Reaction of β-Hydroxyaspartate with L-Aspartate β-Decarboxylase. A New Type of Pyridoxal 5′-Phosphate–Enzyme Inhibition*

Edith Wilson Miles and Alton Meister

ABSTRACT: L-Aspartate β-decarboxylase from *Alcaligenes faecalis* is strongly and competitively inhibited by both *threo-* and *erythro*-β-hydroxyaspartate. The enzyme decarboxylates these amino acids at very slow rates to yield serine; however, the rate of CO_2 liberation exceeds that of serine formation. Incubation of the enzyme with β-hydroxyaspartate leads to a shift of the maximum absorbancy from 360 to 325 mμ; subsequent addition of α-keto acids does not affect the absorbancy suggesting that this form of the enzyme does not contain pyridoxamine 5′-phosphate. Sodium borohydride reduced the holoenzyme in the absence but not in the presence of β-hydroxyaspartate, suggesting that the β-hydroxyaspartate derivative of the enzyme is not a Schiff base. The form of the enzyme that absorbs maximally at 325 mμ (E-325) is inactive in the decarboxylation of L-aspartate and in the desulfination of cysteine sulfinate. Studies in which the enzyme was incubated with [^{14}C]β-hydroxyaspartate and then subjected to gel filtration show that E-325 contains a moiety derived from carbon atoms 1 to 3 of β-hydroxyaspartate; this is converted to serine on further incubation. Treatment of E-325 with 2,4-dinitrophenylhydrazine in hot acid solution yields a compound that appears to be the osazone of glycolaldehyde. The findings indicate that β-hydroxyaspartate is decarboxylated to yield an enzyme–partial substrate intermediate, which is in equilibrium with an inactive enzyme complex.

Aspartate β-decarboxylase has been isolated in apparently homogeneous form from *Alcaligenes faecalis* (Novogrodsky *et al.*, 1963; Novogrodsky and Meister, 1964; Wilson and Meister, 1966) and from *Achromobacter* (Wilson, 1963; Wilson and Kornberg, 1963). This enzyme is subject to at least two different types of inhibition by amino acids. Novogrodsky and Meister (1964) found that L-aspartate and a variety of other L-amino acids inactivate the enzyme by transaminating with the enzyme-bound pyridoxal 5′-phosphate to form pyridoxamine 5′-phosphate. α-Keto acids reactivate the enzyme by reversing the transamination reaction. Conversion of the pyridoxal 5′-phosphate–enzyme (λ_{max} 360 mμ) to the pyridoxamine 5′-phosphate–enzyme (λ_{max} 325 mμ) and the reversal of this transformation were demonstrated spectrophotometrically. Wilson and Kornberg (1963) reported that *threo-* and *erythro*-β-hydroxyaspartate are strong competitive inhibitors of L-aspartate β-decarboxylase from *Achromobacter* and produce a change in the maximum absorbancy of the enzyme from 360 to 325 mμ. However, subsequent treatment of the enzyme with α-keto acids does not affect the absorbancy, suggesting that this form of the enzyme does not contain pyridoxamine 5′-phosphate. Sodium borohydride reduced the holoenzyme in the absence but not in the presence of β-hydroxyaspartate, suggesting that the β-hydroxyaspartate derivative is not a Schiff base.

We now report that *threo-* and *erythro*-β-hydroxyaspartate inhibit L-aspartate β-decarboxylase from *A. faecalis* in a similar manner and that both L isomers of β-hydroxyaspartate are slowly decarboxylated to yield L-serine. Kinetic studies of these reactions and of the inhibition produced by β-hydroxyaspartate have been carried out. These investigations and experiments on the chemical nature of the intermediate form of the enzyme that exhibits maximum absorbancy at 325 mμ indicate that a new type of pyridoxal 5′-phosphate–enzyme inhibition is involved.

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Experimental Section

Materials. L-Cysteine sulfinic acid, *threo*-β-hydroxy-DL-aspartic acid, *erythro*-β-hydroxy-DL-aspartic acid, pyridoxal 5′-phosphate, and pyruvic acid were purchased from California Corp. for Biochemical Research, Los Angeles, Calif. Glyoxylic acid, glycolaldehyde, and α-ketoglutaric acid were obtained from Sigma Chemical Co., St. Louis, Mo. L-Aspartate and glycine were products of Mann Research Laboratories, New York, N. Y. Uniformly labeled [^{14}C]glycine (75 c/mole) was obtained from New England Nuclear Corp., Boston, Mass. Uniformly labeled sodium [^{14}C]glyoxylate (4.7 c/mole) was obtained from Nuclear-Chicago Corp., Des Plaines, Ill. *threo*-β-Methyl-DL-aspartic acid and *erythro*-β-methyl-DL-aspartic acid (92% *erythro*, 8% *threo*) were kindly supplied by Dr. H. A. Barker of the University of California (Berkeley). The β-hydroxy-β-methyl-DL-aspartic acid diastereoisomers were synthesized from cupric glycinate and sodium pyruvate by the modification by Kornguth and Sallach (1960) of the method of Benoiton *et al.* (1959). The *erythro* and *threo* DL isomers were separated, isolated, and identified as described by Jenkins (1961). The [^{14}C]*erythro*- and -*threo*-β-hydroxy-DL-aspartate isomers were synthesized from sodium glyoxylate and cupric glycinate, and isolated and identified by the above methods. Two preparations of [^{14}C]*threo*-β-hydroxyaspartate were made. These products were found to be contaminated with about 15% of an acidic, ninhydrin-negative component which was removed by application of the material to a column of Dowex 50 (H^+) and elution of the amino acid with 2 N NH_4OH. Preparation A was obtained from uniformly labeled [^{14}C]glycine and glyoxylate. Preparation B was obtained from uniformly labeled [^{14}C]glyoxylate and glycine; the reaction mixture was allowed to stand at 4° for 6 days (preparation A was placed at 4° for 1 day). The amount of ^{14}C in carbon atom 4 of the labeled *threo*-β-hydroxy-DL-aspartate preparations was determined by enzymatic β-decarboxylation (see below); the CO_2 formed was collected in scintillation vials containing Hyamine solution as described below (system II). Sufficient enzyme was added to effect complete decarboxylation of the susceptible isomer. When preparations A and B were treated in this manner, 2.6 and 14.8%, respectively, of the radioactivity was released as CO_2. Assuming that only the L isomer is decarboxylated and that the labeling of carbon atoms 3 and 4 and of carbon atoms 1 and 2 are equivalent, it may be concluded that carbon atom 4 contains 5.2 and 29.6% of the radioactivity present in preparations A and B, respectively. Some randomization of the ^{14}C in the course of these syntheses is to be expected because of the occurrence of nonenzymatic transamination between glyoxylate and glycine (Fleming and Crosbie, 1960). More randomization was found with preparation B, which had been allowed to stand at 4° for a longer period (6 days) than preparation A (1 day). The concentration of [^{14}C]β-hydroxyaspartate in solution was determined by the ninhydrin method of Rosen (1957). The purity of the purchased and synthesized diastereoisomers of β-hydroxyaspartate was checked by electrophoresis at pH 3.4 in the system described by Jenkins (1961). No contaminants were detected after treatment with ninhydrin or by scanning of the radioactive strips (see below) under conditions of overloading where a 1% contaminant could be readily detected.

Methods. DETERMINATION OF ENZYME ACTIVITY. Holo-L-aspartate β-decarboxylase was isolated from *A. faecalis* by the method of Wilson and Meister (1966). The preparation used for most of the experiments contained 10 mg of protein/ml and had a specific activity of 2000–4000 units/mg. Enzyme activity was determined as described previously by the colorimetric determination of sulfite liberated from L-cysteine sulfinic acid, which is a substrate for aspartate β-decarboxylase (Wilson and Meister, 1966; Soda *et al.*, 1964). Protein was determined by the procedure of Lowry *et al.* (1951) using crystalline bovine serum albumin as a standard. Spectra of the enzyme were obtained in a Cary Model 14 spectrophotometer. The pyridoxal 5′-phosphate content of the enzyme was determined by the method of Wada and Snell (1961). In the determination of the enzymatic formation of serine from β-hydroxyaspartate, the enzyme at a concentration of about 5 mg/ml in 0.1 M sodium acetate (pH 5.0 or 7.0) was incubated with [^{14}C]β-hydroxyaspartate. The reactions were terminated by adding a one-half volume of 2 N perchloric acid; the denatured protein was removed by centrifugation and the perchloric acid was precipitated from the supernatant solution as potassium perchlorate by adding 2 N KOH to pH 7. One-half of the supernatant solution was applied in bands 5 cm from one end of 3 × 30.5 cm strips of Whatman No. 3MM paper and subjected to electrophoresis in 0.02 M potassium phosphate (pH 7.5) for 2 hr at 131 v/cm. The radioactive areas on the paper strips were located and counted with a Nuclear-Chicago Bio-span 1002 strip counter, making appropriate corrections for background. In some experiments the radioactive areas on paper strips were located with a Vanguard Autoscanner 880, and counted in a scintillation counter in vials containing toluene–phosphor by the method of Wang and Jones (1959).

Radioactive CO_2 was absorbed in 0.1–0.5 ml of a Hyamine hydroxide solution in 1 M methanol by the method of Passmann *et al.* (1956) and counted by the method of Wang and Jones (1959). Experiments in which CO_2 was trapped were carried out in three ways. In system I vessels manufactured by Bonus Laboratories, Boston, Mass., were used. The reaction mixture (0.1 ml) was placed in the center well of the vessel and 0.1 ml of Hyamine solution and a piece of filter paper were placed in the outside chamber. The vessel was closed with a serum cap; after the reaction was terminated by adding 0.05 ml of 2 N $HClO_4$ through the cap with a needle and syringe, the apparatus was allowed to stand at 37° overnight. The Hyamine and filter paper were quantitatively transferred to a scintillation vial with the toluene–phosphor. In system II the reaction mixture was added to a polyethylene

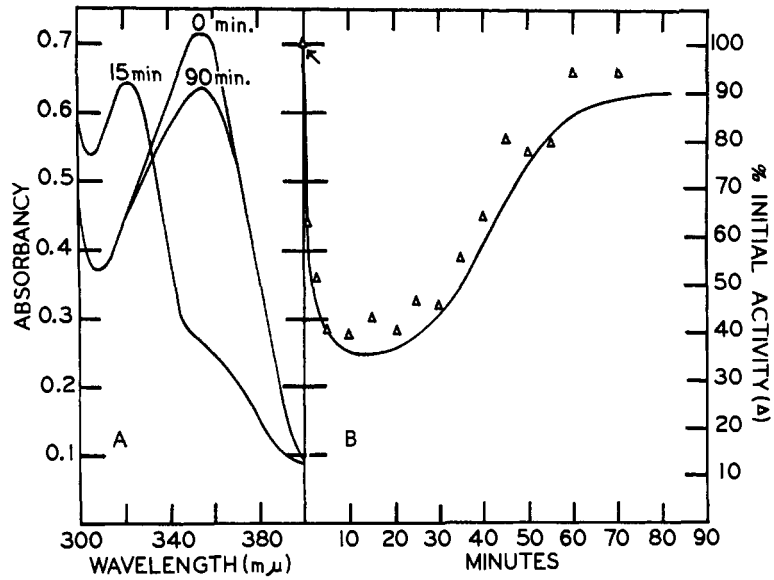


FIGURE 1: The effect of *threo*-β-hydroxyaspartate on the spectrum and activity of L-aspartate β-decarboxylase. L-Aspartate β-decarboxylase (4.8 mg/ml in 0.1 M sodium acetate (pH 6.7) containing 0.065 μmole of bound pyridoxal 5′-phosphate/ml) was treated with *threo*-β-hydroxy-DL-aspartate to give a final concentration of 1 μmole/ml. Spectra were recorded (22°) at 0, 15, and 90 min (A) and the absorbancy at 360 mμ was recorded continuously (B, solid line). Aliquots (0.01 ml) were removed at intervals and added to assay mixtures containing sodium acetate (pH 5.0, 35 μmoles), sodium α-ketoglutarate (0.5 μmole), and L-cysteine sulfinate (50 μmoles) in a final volume of 1.5 ml, 37°. The reaction was terminated after 1.0 min by adding 0.5 ml of 50% trichloroacetic acid, and the sulfite formed was determined. Activity is expressed as per cent of the enzyme activity before adding *threo*-β-hydroxyaspartate (Δ). The arrow indicates the initial activity and absorbancy at 360 mμ.

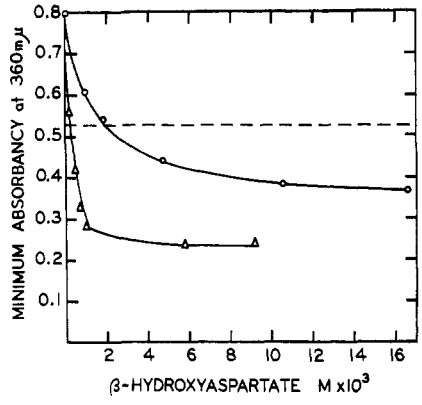


FIGURE 2: The effect of the initial concentration of *threo*- and *erythro*-β-hydroxyaspartate on the maximum decrease of the absorbancy of L-aspartate β-decarboxylase at 360 mμ. L-Aspartate β-decarboxylase (5 mg/ml in 0.1 M sodium acetate (pH 5.0) containing 0.08 μmole of bound pyridoxal 5′-phosphate/ml) was treated with various concentrations of *threo*- or *erythro*-β-hydroxyaspartate. The absorbancy at 360 mμ was followed until minimum values were reached; these values are plotted against the initial concentrations of *threo*-β-hydroxyaspartate (Δ) or *erythro*-β-hydroxyaspartate (○). The dotted line indicates the half-maximal decrease in absorbancy at 360 mμ.

beaker (size 00) placed inside a polyethylene scintillation vial which also contained 0.5 ml of Hyamine solution. The reaction was terminated by adding $HClO_4$ through a hole in the cap which was then sealed. CO_2 absorption was essentially complete after incubation at 25° overnight. The beaker was then removed and toluene–phosphor was added directly to the vial. In system III a small Warburg flask (volume, 5 ml) was used and a piece of filter paper and Hyamine solution were placed in the center well. In certain experiments, aliquots (0.01 or 0.1 ml) were plated on planchets and counted in a gas-flow counter.

GEL FILTRATION. A column containing Sephadex G-25 (coarse) was prepared in a 25-ml buret (height, 36 cm; volume, 25 ml) and equilibrated with 0.01 M potassium phosphate buffer at pH 8.0. A sample (1.0 ml) was applied to the top of the column and then eluted with buffer at a flow rate of 2 ml/min. The eluate was collected in 1-ml fractions, which were assayed for absorbancy at 280 mμ and radioactivity.

Results

Reaction of β-Hydroxyaspartate with the Enzyme; Effects on the Spectrum and on Cysteine Sulfinate Desulfinase Activity. When *threo*-β-hydroxy-DL-aspartate was added to the enzyme at a concentration

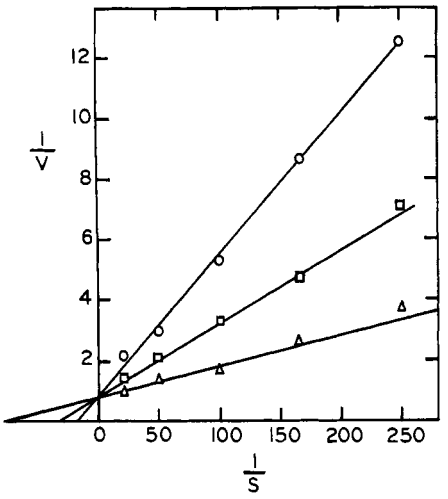


FIGURE 3: The effects of L-cysteine sulfinate concentration on the desulfinase activity of L-aspartate β-decarboxylase in the absence (Δ) and presence of *threo*-β-hydroxy-DL-aspartate (○) or *erythro*-β-hydroxy-DL-aspartate (□). The reaction mixtures contained sodium acetate (pH 5.0, 35 μmoles), sodium α-ketoglutarate (0.5 μmole), pyridoxal 5′-phosphate (0.5 μmole), enzyme (0.025 mg), L-cysteine sulfinate (6–700 μmoles; [S], 0.004–0.466 M), and *threo*-β-hydroxy-DL-aspartate (0.5 μmole) or *erythro*-β-hydroxy-DL-aspartate (5.0 μmoles) in a final volume of 1.5 ml. Aliquots (0.025 ml) were removed after incubation for 10 min at 37° and added directly to 5 ml of the color reagent used for the determination of SO_2.

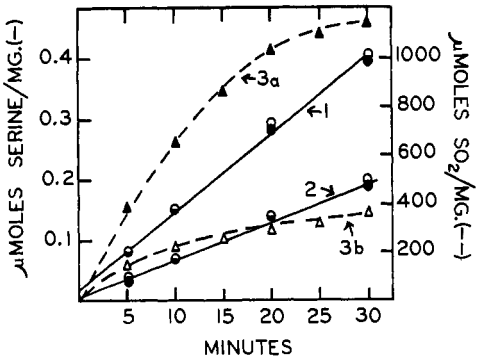


FIGURE 4: Activity of L-aspartate β-decarboxylase toward L-cysteine sulfinate and the *threo* and *erythro* forms of β-hydroxyaspartate in the presence and absence of α-ketoglutarate. The reaction mixtures used for the determination of β-hydroxyaspartate decarboxylation contained sodium acetate (pH 5.0, 2.5 μmoles), enzyme (0.1 mg), [^{14}C]β-hydroxy-DL-aspartate (0.1 μmole, 10^5 cpm), and sodium α-ketoglutarate (0.05 μmole) in a final volume of 0.1 ml; after incubation at 37°, the analyses were performed as described under Methods. Curve 1: *threo*-β-hydroxy-DL-aspartate. Curve 2: *erythro*-β-hydroxy-DL-aspartate. Curve 3a: L-cysteine sulfinate plus α-ketoglutarate. Curve 3b: L-cysteine sulfinate (α-ketoglutarate omitted). Closed symbols indicate the presence of α-ketoglutarate and the open symbols indicate its absence. The assay mixtures used for the determination of desulfinase activity contained sodium acetate (pH 5.0, 35 μmoles), sodium α-ketoglutarate (0.5 μmole), enzyme (0.05 mg), and L-cysteine sulfinate (50 μmoles) in a final volume of 1.5 ml. Aliquots (0.005 ml) were removed at various intervals after incubation at 37° and added directly to 5 ml of the color reagent used for the determination of SO_2.

that was only about 15 times higher than that of enzyme-bound pyridoxal 5′-phosphate, the spectral changes recorded in Figure 1 were obtained. These studies show that the form of the enzyme that exhibits maximum absorbancy at 325 mμ disappears slowly and that the original spectrum returns. At the end of the reaction (100 min after addition of *threo*-β-hydroxy-DL-aspartate), the mixture was deproteinized, desalted, and examined by electrophoresis as described under Methods; about equal amounts of *threo*-β-hydroxyaspartate and serine were found. The serine band was eluted and further identified by reaction with chromotropic acid of the formaldehyde formed by the periodate reaction (Frisell and Mackenzie, 1958).[1] Treatment of the serine with D-amino acid oxidase showed that it contained less than 1% D-serine (Srinivasan *et al.*, 1965).[1] These findings indicate that close to 50% (presumably the L isomer) of *threo*-β-hydroxy-DL-aspartate is converted to L-serine.

The shape of the curve in Figure 1B, which is similar to those found by Chance (1943) in studies on enzyme–substrate intermediates, suggests that the 325 mμ absorbing form is an enzyme–substrate intermediate which breaks down slowly to yield serine. The data given in Figure 1B show that the desulfinase activity of the enzyme is proportional to the amount of the enzyme present in the 360 mμ absorbing form, and therefore suggest that inhibition of desulfinase activity by β-hydroxyaspartate is directly related to the formation of an enzyme-bound intermediate.

The experiments described in Figure 2 show that the maximum decrease in absorbancy of the enzyme at 360 mμ is dependent on the initial concentration of *threo*- or *erythro*-β-hydroxyaspartate. From these data one can estimate that the initial concentrations of *threo*- and *erythro*-β-hydroxyaspartate required for half-maximal reduction of absorbancy at 360 mμ (dotted line) are 3.5 and 23 × 10^{-4} M, respectively; thus the enzyme exhibits a much higher affinity for the *threo* form. At initial concentrations of *threo*- and *erythro*-β-hydroxyaspartate which give the same maximum decrease in absorbancy of the enzyme at 360 mμ, the time required for this decrease with the *erythro* form is about three times greater than that required with the *threo* form.

[1] We are indebted to Dr. John J. Corrigan for assistance in carrying out these studies.

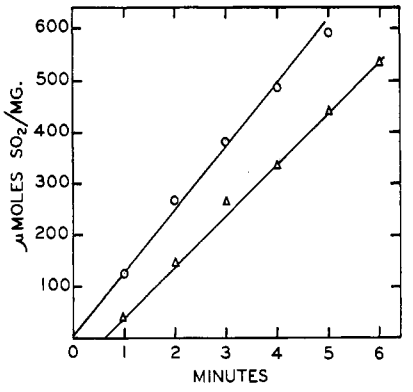


FIGURE 5: The effect of preincubation of the enzyme with *threo*-β-hydroxyaspartate on the time course of the reaction catalyzed by L-aspartate-β-decarboxylase. The enzyme was treated with 1 mM *threo*-β-hydroxyaspartate as in Figure 1. After 10 min the minimal absorbancy at 360 mμ was attained and an aliquot (0.01 ml) was added to the standard assay mixture (as carried out above; see Figure 1), and incubated at 37°. Aliquots (0.01 ml) were removed at 1-min intervals and added directly to 5 ml of the color reagent (Δ). Untreated enzyme was used as the control (○).

Effects of β-Hydroxyaspartate and Other Aspartate Analogs on Activity. Figure 3 describes the effect of substrate concentration on the desulfinase activity of the enzyme in the absence and presence of β-hydroxyaspartate; the data are plotted according to the method of Lineweaver and Burk (1934). The Michaelis constant (K_m) for L-cysteine sulfinate was calculated to be 0.012 M, a value that is close to 0.017 M, which was reported by Soda *et al.* (1964). The findings show that *threo*-β-hydroxy-DL-aspartate and *erythro*-β-hydroxy-DL-aspartate are competitive inhibitors of L-aspartate β-decarboxylase, with K_I values of 0.89 and 24 × 10^{-4} M, respectively. Similar results were obtained by Wilson and Kornberg (1963) with the enzyme from *Achromobacter* except that the respective K_I values were approximately ten times lower than found with the present enzyme.

The effect of α-ketoglutarate on the activity of the enzyme toward L-cysteine sulfinate and the *threo* and *erythro* forms of β-hydroxy-DL-aspartate (in the absence of added pyridoxal 5′-phosphate) is described in Figure 4. α-Ketoglutarate was stimulatory only with L-cysteine sulfinate, indicating that this substrate, but not β-hydroxyaspartate, transaminates with the enzyme. The maximum reaction rate with L-cysteine sulfinate was about 4000 times greater than that with *threo*-β-hydroxy-DL-aspartate. Although the rate with *threo*-β-hydroxyaspartate in these experiments is about twice that found with the *erythro* isomer, it should be noted that the concentration of the *erythro* isomer used here was lower than its K_I value. The maximum rates of decarboxylation of the two isomers are about the same, but as stated above, the enzyme exhibits a much higher affinity for the *threo* isomer.

Several other aspartate analogs (β-methylaspartate, β-methyl-β-hydroxyaspartate, and α-aminotricarballylate) were tested as substrates and inhibitors of the enzyme. The only analog that was significantly decarboxylated (other than β-hydroxyaspartate) was *erythro*-β-methyl-DL-aspartate; the formation of α-aminobutyrate was shown by paper electrophoresis. The β-methylaspartate isomers were also tested as inhibitors by preincubating the enzyme with the amino acids in the absence and presence of α-ketoglutarate

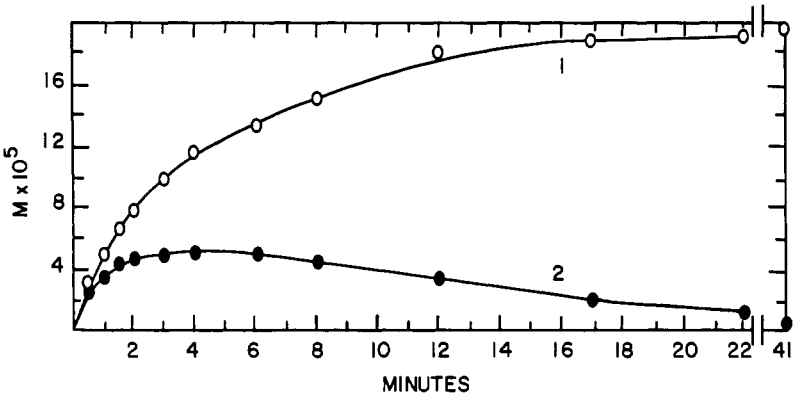


FIGURE 6: The rate of formation of L-[^{14}C]serine from [^{14}C]*threo*-β-hydroxy-DL-aspartate. L-Aspartate β-decarboxylase (5 mg/ml in 0.1 M sodium acetate buffer, pH 5.0) containing 8 × 10^{-5} M bound pyridoxal 5′-phosphate was treated with [^{14}C]*threo*-β-hydroxy-DL-aspartate (preparation A) (40 × 10^{-5} M). The absorbancy at 360 mμ was recorded in a Cary 14 spectrophotometer at 22°, and aliquots (0.1 or 0.2 ml) were removed at intervals for the determination of serine as described under Methods. The total radioactivity counted on each paper electrophoresis strip was between 14,000 and 30,000 cpm. The values were counted for the distribution of ^{14}C in the β-hydroxyaspartate (see Materials). The concentration of the enzyme intermediate was calculated from the absorbancy at 360 mμ as described in the text. Curve 1: serine. Curve 2: enzyme intermediate.

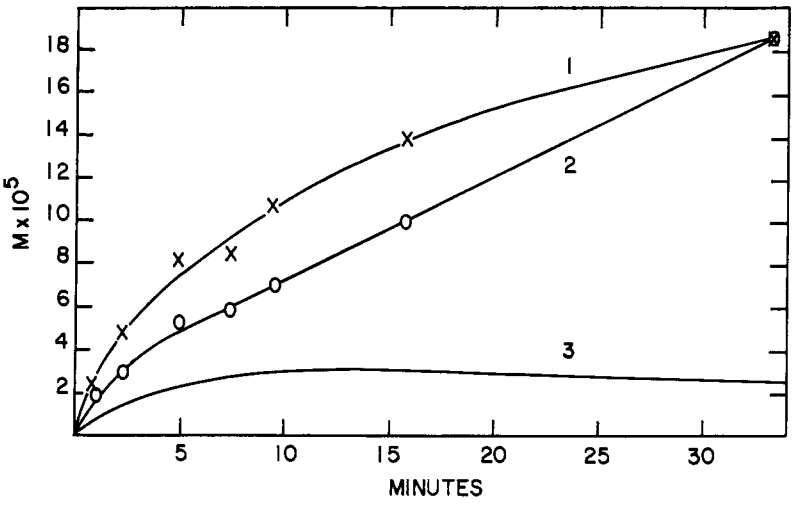


FIGURE 7: The rate of formation of [^{14}C]serine (curve 2) and $^{14}CO_2$ (curve 1) from [^{14}C]*threo*-β-hydroxy-DL-aspartate. Aliquots (0.1 ml) of L-aspartate β-decarboxylase (5 mg/ml in 0.1 M sodium acetate buffer, pH 7.0) containing 6.2×10^{-5} M bound pyridoxal 5′-phosphate were placed in CO_2 collection vessels (type I) and treated with [^{14}C]*threo*-β-hydroxy-DL-aspartate (preparation B) to give an initial concentration of 3.72×10^{-4} M. The absorbancy at 360 mμ of 1.0 ml of enzyme treated in the same way at 25° was followed simultaneously, and the reaction in each vessel was terminated at the indicated time by addition of perchloric acid. CO_2 and serine were determined as described under Methods. The serine and β-hydroxyaspartate areas on electrophoretograms were separately counted with a scintillation counter. The values were corrected in accordance with the labeling pattern of the [^{14}C]β-hydroxyaspartate (see Materials). The concentration of the enzyme intermediate (curve 3) was calculated as described in the text.

followed by assay with cysteine sulfinate in the presence of α-ketoglutarate. Prevention of inhibition by α-ketoglutarate under these conditions represents a sensitive assay for transamination between amino acid and enzyme (Novogrodsky and Meister, 1964). *erythro*-β-Methyl-DL-aspartate produced inhibition that was significantly prevented by α-ketoglutarate; the *threo* isomer was much less active than the *erythro* form.

Properties of the Enzyme Derivative that Absorbs Maximally at 325 mμ. The activity of the enzyme toward L-cysteine sulfinate before and after preincubation with 1 mM *threo*-β-hydroxyaspartate is described in Figure 5. After such preincubation there was a lag of about 0.6 min before a rate was established that was close to that exhibited by the untreated enzyme. The rate of enzymatic decarboxylation of *threo*-β-hydroxyaspartate under these conditions is 0.013 μmole/mg per min (Figure 4) and the pyridoxal 5′-phosphate content of the enzyme is 0.0135 μmole/mg. The observed lag period is, therefore, consistent with the time required for the turnover of an enzyme–substrate intermediate.

The findings suggest that the enzyme derivative which absorbs at 325 mμ is an enzyme–substrate intermediate since it disappears when all of the susceptible substrate has been decarboxylated (Figure 1), and since the rate and maximum extent of its formation are dependent on substrate concentration (Figure 2). If the form absorbing at 325 mμ were an *obligatory* enzyme–substrate intermediate, the rate of product formation would be expected to be proportional to the concentration of this intermediate (as first shown by Chance, 1943), and the initial course of product formation should exhibit a lag period equal to the time necessary for the maximal decrease in absorbancy at 360 mμ (and its corresponding increase at 325 mμ). Figure 6 describes an experiment in which the time course of serine formation from *threo*-β-hydroxyaspartate was followed and compared with the formation of the enzyme intermediate which absorbs maximally at 325 mμ. The concentration of the enzyme intermediate was calculated from the known concentration of enzyme-bound pyridoxal 5′-phosphate (8×10^{-5} M) and from the observed decrease in absorbancy at 360 mμ. It is assumed that all of the pyridoxal 5′-phosphate is converted to the intermediate in the presence of a saturating concentration of *threo*-β-hydroxyaspartate (under these conditions the net decrease in absorbancy at 360 mμ is 0.61; *cf.* Figure 2), and that therefore the concentration of enzyme intermediate is proportional to the decrease in absorbancy at 360 mμ in accordance with the following relation

$$\text{enzyme intermediate (moles/l.)} = \frac{\Delta A_{360\,m\mu} \times 8 \times 10^{-5}}{0.61}$$

As shown in Figure 6, the rate of serine formation does not exhibit a lag period. The initial rate is maximal and is observed before the maximal formation of the enzyme intermediate occurs; thus, the rate of product

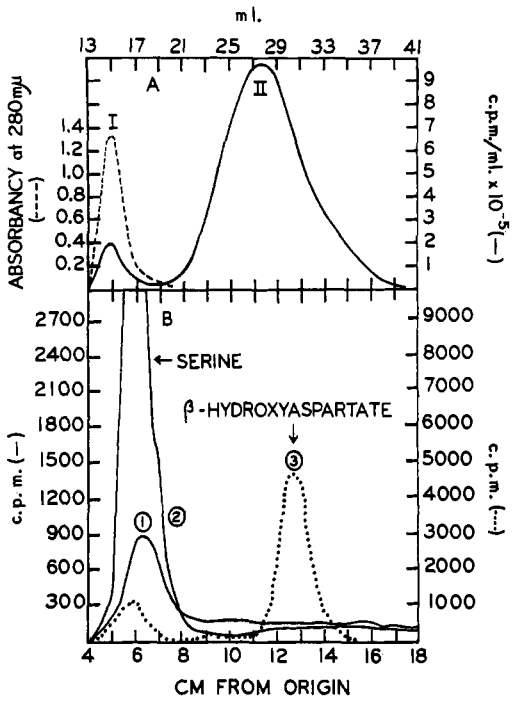


FIGURE 8: Isolation of the enzyme derivative absorbing at 325 mμ by gel filtration. (A) Elution of the enzyme derivative from a column of Sephadex. Aspartate-β-decarboxylase (0.9 ml containing 4.6 mg/ml and 6.5×10^{-5} M pyridoxal 5′-phosphate) was treated with 0.09 ml of [^{14}C]*threo*-β-hydroxyaspartate (preparation A, 0.9 μmole, 900,000 cpm) for 4 min at 22°; this solution was then applied to the top of a column of Sephadex G-25 and eluted as described under Methods. The absorbancy at 280 mμ and radioactivity of each fraction were determined. Fractions 14–17, which contained a total of 44,200 cpm, were combined and deproteinized with $HClO_4$; the perchlorate was removed as described under Methods. The solution was desalted by applying it to a column of Dowex 50 (H^+; 1 ml) followed by elution with 2 N NH_4OH; after concentration of the eluate, it was subjected to electrophoresis as described under Methods. (B) Electrophoresis of the amino acids, obtained from fractions I and II. Curve 1: fraction I, immediately after emerging from the column. Curve 2: fraction I, after incubation at 25° for 30 min. Curve 3: fraction II.

formation is not proportional to the concentration of intermediate.

Figure 7 describes a similar experiment in which the rates of CO_2 and serine production from [^{14}C]-*threo*-β-hydroxyaspartate were measured; simultaneous spectral studies were carried out. These data show that CO_2 is produced more rapidly than serine. The difference between the molar concentrations of CO_2 and serine formed are close to the estimated molar concentration of the enzyme intermediate which absorbs maximally at 325 mμ. These findings suggest that the enzyme intermediate contains a moiety derived (after decarboxylation) from β-hydroxyaspartate.

Isolation of the Enzyme Derivative that Absorbs Maximally at 325 mμ. The low rate of turnover of the postulated enzyme–product intermediate suggested that it might be possible to isolate it by gel filtration. Figure 8 describes the results of an experiment in which the enzyme was incubated with [^{14}C]*threo*-β-hydroxyaspartate and then separated from low molecular weight substances by passage through a Sephadex G-25 column. The total amount of radioactivity associated with the protein peak (A, I) was about 75% (calculated on a molar basis) of that of the pyridoxal 5′-phosphate present, indicating that relatively little dissociation occurred during the separation procedure. When fraction I was immediately deproteinized with perchloric acid, followed by removal of the perchloric acid as potassium perchlorate and desalting on a column of Dowex 50 (H^+), about 30% of the original ^{14}C present in fraction I was recovered. All of this ^{14}C was shown by electrophoresis to be [^{14}C]serine (Figure 8B, curve 1); no β-hydroxyaspartate was found. Similar study of fraction II (Figure 8A) showed that 80% of the ^{14}C present was associated with β-hydroxyaspartate and 20% with serine (Figure 8B, curve 3). Fraction I initially exhibited a spectrum with a maximum at 325 mμ and a shoulder at 360 mμ. After incubation of this fraction for 30 min at 25°, the maximum absorbancy shifted from 325 to 360 mμ. When the solution was then deproteinized and processed as described above, more than 80% of the radioactivity originally associated with fraction I was recovered as serine (Figure 8B, curve 2). These data indicate that most of the radioactivity bound to the form of the enzyme absorbing at 325 mμ represents neither β-hydroxyaspartate nor serine, but is associated with another compound which is converted to serine by incubation at 25°.

Table I describes experiments carried out in an effort to further elucidate the nature of the enzyme-bound precursor of serine. In these studies, the enzyme was incubated with labeled *threo*-β-hydroxyaspartate, after which gel filtration was carried out as described in Figure 8A. Aliquots of the enzyme intermediate that absorbs maximally at 325 mμ (E-325) were used for the determination of total radioactivity and serine, and were treated with 2,4-dinitrophenylhydrazine as described below. The same determinations were carried out after incubation for 60 min at 25° (E-360). As indicated in Table I, there was no substantial change in the total radioactivity present after incubation; in expt 2, it was established that there was no evolution of $^{14}CO_2$ during the incubation. The difference between the total amount of radioactivity associated with the enzyme in expt 1 (60,500 cpm) and 2 (41,700 cpm) is consistent with the labeling patterns of the preparations of [^{14}C]*threo*-β-hydroxyaspartate (see Materials) on the basis that β-decarboxylation had occurred before the enzyme intermediate was isolated. On treatment of the $E_{325\,m\mu}$ form of the enzyme with 2,4-dinitrophenylhydrazine in acid solution at

TABLE I: Properties of the Enzyme Derivative Isolated by Gel Filtration.[a]

Experiment	Enzyme Derivative (cpm) E-325	E-360
1. [^{14}C]*threo*-β-Hydroxyaspartate (A)		
Total ^{14}C present	60,500	61,300
^{14}C as 2,4-dinitrophenylhydrazine derivative	26,300 (31,800)[b]	1,960
^{14}C as serine	20,000	60,000
2. [^{14}C]*threo*-β-Hydroxyaspartate (B)		
Total ^{14}C present	41,700	37,900[c]
^{14}C as 2,4-dinitrophenylhydrazine derivative	29,400 (29,800)[b]	3,480
^{14}C as serine	13,000	47,000[d]

[a] The reaction mixtures consisted of enzyme (5 mg), [^{14}C]*threo*-β-hydroxy-DL-aspartate (0.08 μmole; 2 × 10^6 cpm; expt 1, preparation A; expt 2, preparation B), and sodium acetate buffer (pH 5.0; 100 μmoles) in a final volume of 1.0 ml. After incubation at 25° for 2 min, fractionation was carried out on columns of Sephadex G-25 as described in Figure 8A. The fractions containing the enzyme were combined; aliquots were taken immediately for analysis, and the remainder of the fraction was incubated for 1 hr at 25° and then analyzed. Treatment with 2,4-dinitrophenylhydrazine was carried out as follows. The aliquot was treated for 30 min with one-tenth volume of 0.2% 2,4-dinitrophenylhydrazine in 2 N HCl at 100°; after cooling, the solution was extracted with ethyl acetate. Serine was determined after deproteinization with 75% ethanol followed by paper electrophoresis as described under Methods. In expt 2, incubation at 25° was carried out in a Warburg flask containing Hyamine solution to trap evolved $^{14}CO_2$ as described under Methods. [b] Calculated for loss of carbon atoms 1 and 4. [c] No $^{14}CO_2$ was formed during the incubation (60 min, 25°) required to convert E-325 to E-360. [d] This sample was counted under conditions in which a 15% error was possible; the other determinations are subject to about 5% error.

100° a nonacidic, ethyl acetate extractable derivative was obtained which contained a substantial proportion of the initial radioactivity. The 2,4-dinitrophenylhydrazine derivative is tentatively considered to be the osazone of glycolaldehyde on the basis of the following evidence. The $E_{325\,m\mu}$ and $E_{360\,m\mu}$ forms of the enzyme were isolated in an experiment identical with that described in Table I except that the enzyme was treated with 10 μmoles of *threo*-β-hydroxy-DL-aspartate prior to separation by gel filtration. Equal aliquots (1.5 mg in 1.5 ml) of the two forms were treated for 30 min with 0.1 ml of 0.2% 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid at 100°; after cooling, the solutions were made alkaline and extracted with 1 ml of isoamyl alcohol. The extract obtained from the $E_{325\,m\mu}$ form of the enzyme was purple while that obtained from the $E_{360\,m\mu}$ form was yellow-brown. The difference spectrum ($E_{325\,m\mu}$ form − $E_{360\,m\mu}$ form) exhibited a maximum absorbancy at 560 mμ and a shape close to that given by an authentic sample of the osazone of glycolaldehyde prepared under the same conditions. Such a spectrum is characteristic of osazones prepared in this manner (Smyrniotis *et al.*, 1958; Friedemann and Haugen, 1943). The findings suggest that the enzyme-bound 3-carbon atom precursor of serine is converted to glycolaldehyde under the conditions employed. The amount of radioactivity found in the 2,4-dinitrophenylhydrazine derivative is consistent with the loss of carbon atom 1 in both experiments (Table I).

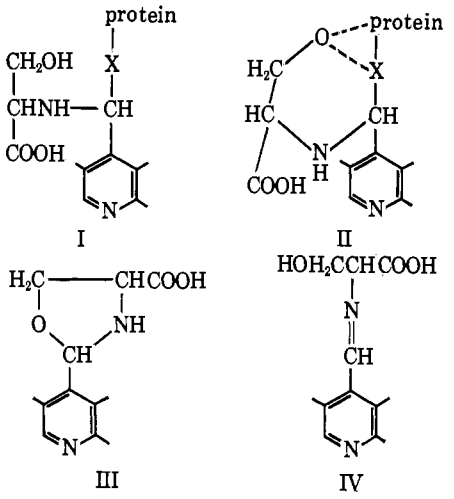
Discussion

The present study shows that one isomer (presumably the L isomer) of β-hydroxy-DL-aspartate is decarboxylated by aspartate-β-decarboxylase to yield L-serine. The maximum rates of decarboxylation of the *threo* and *erythro* forms of β-hydroxyaspartate are similar (about 0.02% of the maximum rates of the decarboxylation of aspartate and the desulfination of cysteine sulfinate), but the enzyme exhibits a greater affinity for the *threo* form. Both stereoisomers of β-hydroxyaspartate are effective competitive inhibitors toward cysteine sulfinate and aspartate.

When the enzyme is incubated with β-hydroxyaspartate, the maximum absorbancy shifts from 360 to 325 mμ. The form of the enzyme that exhibits maximum absorbancy at 325 mμ (E-325) is inactive toward aspartate and cysteine sulfinate (Figure 1). The findings indicate that E-325 is not the free pyridoxamine phosphate form of the enzyme, but that it is a pyridoxal phosphate–amino acid derivative. The absence of a lag period in the formation of serine and the fact that serine formation is not proportional to the concentration of E-325 indicate that E-325 is not an obligatory intermediate in the decarboxylation of β-hydroxyaspartate. Nevertheless, the extent of E-325 formation is dependent upon the initial concentration of β-hydroxyaspartate and E-325 disappears at the end of the reaction at which time 50% of the added β-hydroxy-DL-aspartate is found as L-serine. It therefore may be concluded that E-325 is produced in a reversible side reaction and that it is in equilibrium with one of the normal enzyme–substrate intermediates. There would appear to be two possible general types of such side reactions, *i.e.*, those leading to ES* or EP* as indicated in the following scheme.

$$\begin{array}{ccccccc} E + S & \rightleftharpoons & ES & \xrightleftharpoons{-CO_2} & EP & \rightleftharpoons & E + P \\ & & \downarrow\uparrow & & \downarrow\uparrow & & \\ & & ES^* & & EP^* & & \end{array}$$

CHART I



Three experimental approaches indicate that E-325 is of type EP*. Thus, the rate of carbon dioxide liberation is greater than that of serine formation (Figure 7). Furthermore, the amount of radioactivity associated with E-325 isolated by gel filtration after incubation of the enzyme with two types of labled [^{14}C]*threo*-β-hydroxyaspartate is consistent with the loss of carbon atom 4. Finally, conversion of E-325 to E-360 is not associated with formation of carbon dioxide (Table I). The findings therefore indicate that E-325 contains a three-carbon moiety derived from carbon atoms 1 to 3 of β-hydroxyaspartate; this moiety is converted to serine. Attempts to obtain E-325 by incubation of the enzyme with L-serine have not been successful. Thus, when the enzyme was incubated with 0.1 M L-serine, there was a gradual decrease in absorbancy at 360 mμ associated with formation of a maximum at 325 mμ; this shift was slowly reversed on addition of 0.01 M sodium α-ketoglutarate. The findings indicate that the enzyme transaminates with L-serine (as reported earlier by Novogrodsky and Meister, 1964).

It appears that the formation of E-325 is associated with the decarboxylation reaction and the presence of the β-hydroxyl group. It is of interest that β-methylaspartate does not exhibit inhibition of the type observed with β-hydroxyaspartate. It is conceivable that decarboxylation is associated with increase in reactivity of the β-hydroxyl group. Although E-325 ultimately yields serine (probably through a Schiff base intermediate), it would appear that, at least under the conditions employed, reduction by borohydride was not favored.[2] The present findings indicate that the three-carbon moiety attached to E-325 is converted in acid to glycolaldehyde by α-decarboxylation. Although several plausible mechanisms for the formation of E-325 and glycolaldehyde may be considered, additional information is needed to explain the observations. We may tentatively consider structures I–III for the E-325 form (see Chart I). Structure I is a substituted aldimine derivative similar to that proposed for phosphorylase (Kent *et al.*, 1958) and previously suggested by Wilson and Kornberg (1963). A structure involving reaction of the hydroxyl oxygen atom with the enzyme must also be considered (II); structure III, the oxazolidine derivative, is analogous to the thiazolidine derivative of pyridoxal phosphate which may be formed nonenzymatically from pyridoxal phosphate and cysteine (Buell and Hansen, 1960). Enzymatic formation of oxazolidine derivatives of pyridoxal phosphate has been suggested by Nishimura and Greenberg (1961) and by Torchinsky and Koreneva (1964) to explain the inhibition of L-threonine dehydrase by L-serine and of glutamate–aspartate transminase by isoserine, respectively. Pyridoxal and pyridoxal phosphate oxazolidines have been prepared from nitrogen-substituted β-amino alcohols (Osbond, 1964). However, detailed chemical studies of the properties of oxazolidine derivatives of pyridoxal or pyridoxal phosphate do not appear to have been reported. Although additional study is needed of the mechanism by which β-hydroxyaspartate inhibits aspartate β-decarboxylase, the data thus far obtained indicate that this substrate combines effectively with the enzyme to yield a partial substrate complex, whose further examination may shed more light on the structure and mechanism of action of this enzyme.

[2] Treatment of the holoenzyme with sodium borohydride at pH 5 reduced the enzyme (Wilson and Kornberg, 1963). However, no loss of pyridoxal phosphate occurred when such treatment was carried out in the presence of β-hydroxyaspartate. In studies in which portions of sodium borohydride were added to E-325 at pH 8 over a period of 30 min to give a final concentration of 1 mg/ml, no evidence for reduction of the enzyme was obtained nor did resolution of the enzyme occur.

References

Benoiton, L., Winitz, M., Colman, R. F., Birnbaum, S. M., and Greenstein, J. P. (1959), *J. Am. Chem. Soc. 81*, 1726.

Buell, M. V., and Hansen, R. E. (1960), *J. Am. Chem. Soc. 82*, 6042.

Chance, B. (1943), *J. Biol. Chem. 151*, 553.

Fleming, L. W., and Crosbie, G. W. (1960), *Biochim. Biophys. Acta 43*, 139.

Friedemann, T. E., and Haugen, G. E. (1943), *J. Biol. Chem. 147*, 415.

Frisell, W. R., and Mackenzie, C. G. (1958), *Methods Biochem. Anal. 6*, 63.

Jenkins, W. T. (1961), *J. Biol. Chem. 236*, 1121.

Kent, A. B., Krebs, E. G., and Fischer, E. H. (1958), *J. Biol. Chem. 232*, 549.

Kornguth, M. L., and Sallach, H. J. (1960), *Arch. Biochem. Biophys. 91*, 39.

Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 56*, 658.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. F., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Miles, E. W., Novogrodsky, A., and Meister, A. (1966),

2nd International Conference on Pyridoxal Catalysis, Moscow, Sept (in press).
Nishimura, J. S., and Greenberg, D. M. (1961), *J. Biol. Chem. 236*, 2684.
Novogrodsky, A., and Meister, A. (1964), *J. Biol. Chem. 239*, 879.
Novogrodsky, A., Nishimura, J. S., and Meister, A. (1963), *J. Biol. Chem. 238*, PC1903.
Osbond, J. M. (1964), *Vitamins Hormones 22*, 367.
Passmann, J. M., Radin, N. S., and Cooper, J. A. D. (1956), *Anal. Chem. 28*, 484.
Rosen, H. (1957), *Arch. Biochem. Biophys. 67*, 10.
Smyrniotis, P. Z., Miles, H. T., and Stadtman, E. R. (1958), *J. Am. Chem. Soc. 80*, 2541.
Soda, K., Novogrodsky, A., and Meister, A. (1964), *Biochemistry 3*, 1450.
Srinivasan, N. G., Corrigan, J. J., and Meister, A. (1965), *J. Biol. Chem. 240*, 796.
Torchinsky, Yu. M., and Koreneva, L. G. (1964), *Biokhimya 29*, 780.
Wada, H., and Snell, E. E. (1961), *J. Biol. Chem. 236*, 2089.
Wang, C. H., and Jones, D. E. (1959), *Biochem. Biophys. Res. Commun. 1*, 203.
Wilson, E. M. (1963), *Biochim. Biophys. Acta 67*, 345.
Wilson, E. M., and Kornberg, H. L. (1963), *Biochem. J. 88*, 578.
Wilson, E. M., and Meister, A. (1966), *Biochemistry 5*, 1166.

Purification and Properties of Two Acid Phosphatase Fractions Isolated from Osmotic Shock Fluid of *Escherichia coli**

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ABSTRACT: An osmotic shock procedure causes the specific release from *Escherichia coli* of a group of hydrolytic enzymes considered to be localized near the surface of the cell. Three distinct acid phosphatase fractions have been purified and examined. One of these, accounting for about 70% of the activity against glucose 6-phosphate of cell extracts, was purified 124-fold from the shock fluid and was homogeneous on disc gel electrophoresis. It had a striking specificity for hexose phosphates, although ribose 5-phosphate was also split at a substantial rate. The second fraction hydrolyzed a number of naturally occurring phosphate monoesters but was most active against *p*-nitrophenyl phosphate. The third fraction also hydrolyzed *p*-nitrophenyl phosphate, but evidence is presented that this represents a new activity for an enzyme previously described in the literature, namely cyclic phosphodiesterase. Growth conditions and media are described by which the concentration of hexose phosphatase may be varied over a 100-fold range.

The acid phosphatase activity of *Escherichia coli* has been incompletely characterized. Rogers and Reithel (1960) described two major phosphatases active at an acid pH but only one of these was extensively purified; this was a nucleoside 2′- or 3′-phosphatase which was virtually inactive against sugar phosphates. Von Hofsten and Porath (1962) purified an acid phosphatase from extracts of *E. coli* and found it to be very active against hexose phosphate monoesters although a complete summary of substrate specificity has not been published. Neu and Heppel (1964, 1965) found that acid phosphatase was among the group of enzymes released into the medium when *E. coli* was converted into spheroplasts by treatment with lysozyme and EDTA, or when cells were subjected to osmotic shock. Osmotic shock provides a convenient first step in the fractionation of released enzymes, capable of giving up to a 30-fold purification compared with sonic extracts.

In the present report we describe two acid phosphatase fractions obtained from shock fluid. One of these was partially purified by Neu and Heppel (1964) and we have added additional steps in order to obtain a preparation that is homogeneous on disc gel electrophoresis. This enzyme is now shown to be highly specific for certain sugar phosphate esters and is referred to as the acid hexose phosphatase. Conditions for its suppression will be discussed. The second fraction differs in its specificity from any acid phosphatase previously described in *E. coli;* it has thus far resisted substantial purification and is designated as the nonspecific acid phosphatase. Evidence is also presented

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