1-Aspartate-α-decarboxylase, an enzyme that catalyzes the formation of β-alanine in Escherichia coli*

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1-Aspartate-α-decarboxylase, an enzyme that catalyzes the production of β-alanine, has been purified to apparent homogeneity from Escherichia coli. The properties of the enzyme are: (a) pH optimum of 6.8 to 7.5, (b) temperature optimum of 55°C, (c) Km for L-α-aspartate of 0.16 mM, and (d) molecular weight of 58,000. The activity of the enzyme is inhibited by reagents (hydroxylamine, phenylhydrazine, and sodium borohydride) that react with carbonyl groups, but no pyridoxal phosphate is present. The compound containing the carboxyl group has been identified as covalently bound pyruvate. Approximately 1 mol of pyruvate was found/mol of enzyme. That the enzyme has a biosynthetic function rather than a catabolic role is indicated by the observations that a mutant (designated as E. coli 99-2) which requires either β-alanine or pantothenic acid for growth contains only trace amounts of enzyme activity, whereas it is present in substantial amounts in the parent strain (E. coli W) and in a spontaneous revertant of the mutant.

Some of the roles of β-alanine in biological processes are well known. This compound is a component of the dipeptides anserine and carnosine, substances which are found in substantial quantities in skeletal muscle of vertebrates (1), and it has been reported to be a cross-linking agent in the cuticles of insects (2). However, probably the most important general function of β-alanine is its use as a substrate for the enzymatic synthesis of pantothenic acid in microorganisms (3-5). Pantothenate is then converted enzymatically to its metabolically active form, coenzyme A (6).

Several reports have appeared concerning the enzymatic synthesis of β-alanine. This substance is known to be produced as a degradation product of uracil in animals (7-9), plants (10), and some microorganisms (11). Rendina and Coon (12) have suggested that it can be synthesized by transamination of malonyl semialdehyde (produced from propionic acid), and indeed there is evidence that such a transamination can occur (13-17). However, the most reasonable possibility for the formation of β-alanine used for the biosynthesis of pantothenic acid and coenzyme A is by α-decarboxylation of aspartic acid. Indirect evidence that aspartate is a precursor of β-alanine has accumulated with the finding that in Escherichia coli the growth inhibitory action of cysteic acid and β-hydroxysaspartic acid can be reversed by either aspartic acid, β-alanine, or pantothenic acid (18, 19). Also, any of these three compounds can reverse the growth inhibition caused by D-serine in E. coli (20, 21) and in a species of Flavobacterium (17).

Several workers have reported on the probable enzymatic conversion of aspartic acid to β-alanine in bacteria (22-26), but the levels of enzymatic activity were all so low that these observations were not pursued further. The only workers, until now, who have investigated this process to any significant extent are Nakano and Kitaoka (27), who partially purified an aspartate-α-decarboxylase from E. coli B and reported that it contains pyridoxal phosphate as a coenzyme. However, the fact that the specific activity of the decarboxylase was greatly increased by growing the cells at an acid pH in the presence of large amounts of aspartate and glutamate over that observed from cells grown at pH 7.0 on minimal medium suggests that the decarboxylase that they studied is an inducible catabolic enzyme. In this paper we report on the discovery, purification, and some properties of another aspartate-α-decarboxylase in E. coli. This enzyme contains a covalently-bound pyruvyl residue as a prosthetic group instead of pyridoxal phosphate, and the evidence indicates that it is not a catabolic enzyme, but rather that it is responsible for the biosynthesis of β-alanine needed for the formation of pantothenic acid and coenzyme A.

MATERIALS AND METHODS

E. coli B, E. coli W (ATCC No. 9637), and Saccharomyces carlsbergensis 4228 were obtained from the American Type Culture Collection. E. coli 99-2 (a β-alanine auxotroph derived from E. coli W) was obtained from Dr. Allan Larabee of Memphis State University. A spontaneous revertant was isolated in this laboratory, Bovine serum albumin, DNAse I, lactate dehydrogenase, various kinds of Sephadex, and calibration proteins for molecular weight determinations were purchased from Sigma Chemical Co.; hydroxylapatite and AG50W X4 from Bio-Rad; Ultrogels from LKB; PM-10 ultrafiltration membranes from Amicon Corp.; Whatman No. 3MM paper and Scintiverse from Fisher Scientific; cellulose thin layer plates from Eastman Kodak Co.; sodium "Hlborohydride (270 mCi/mmol), [1-4C]aspartic acid (184 mCi/mmol), [1,4-14C]aspartic acid (134 mCi/mmol), [1,4,4-14C]aspartic acid (14.6 mCi/mmol), [3,4-14C]aspartic acid (23.6 mCi/mmol), [1,4,4-14C]aspartic acid (18.4 mCi/mmol), [1-14C]alanine (2 mCi/mmol), [3-14C]alanine (2 mCi/mmol), and hyamine hydroxide from New England Nuclear; and [4-14C]phenylhydradine-HCl (5.5 mCi/mmol) from ICN. Other compounds were obtained from commercial sources.

Microbiological Methods—Microbiological assay for the production of pantothenic acid and β-alanine (asparagine omitted from the medium) was as described by Atkin et al. (28). E. coli was grown in minimal medium (29) supplemented with 0.5% glucose and with yeast extract (3 g/liter) and Bacto-peptone (5 g/liter) when a rich medium was used. Cells (harvested at late exponential growth phase) were grown at 37°C with vigorous aeration in 12-liter quantities in 16-liter carboys.

Enzyme Assay—The reaction mixture contained (per 0.5 ml total

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stopped by the addition of 0.05 ml of 50% trichloroacetic acid. The similar 1-mm slices was determined in a scintillation counter (5 mm standard reaction mixtures as a source of enzyme. Radioactivity of activity, the gel was cut into 1-mm slices which were added to on an individual strip (3 cm wide) of Whatman 3 MM paper. After staining with Coomassie blue. To determine the position of enzymatic in the presence of sodium dodecyl sulfate was as described by Weber and Osborn (33). Protein bands on developed gels were located by volume), radioactive zones were located with a Packard Model 7200 Radiochromatogram Scanner. The zone corresponding to \( \beta \)-alanine was identified by comparison with the zone of migration of standard \( \beta \)-L-[\( ^{14} \)C]alanine.

**Electrophoresis on Polyacrylamide Gels**—The method of Davis (31) was used for preparative electrophoresis, and that of Peacock et al. (32) for analytical electrophoresis at pH 7.5 and 9.5; electrophoresis in the presence of sodium dodecyl sulfate was as described by Weber and Osborn (33). Protein bands on developed gels were located by staining with Coomassie blue. To determine the position of enzymatic activity, the gel was cut into 1-mm slices which were added to standard reaction mixtures as a source of enzyme. Radioactivity of similar 1-mm slices was determined in a scintillation counter (5°C for 3 to 12 h).

**Miscellaneous Methods**—Protein was estimated by the method of Lowry et al. (34) with bovine serum albumin as standard, or from the ratio of absorbance at 260 and 280 nm (35). Preparative isoelectric focusing was carried out according to Vesterburg (36). Reduction in volume of enzyme preparations was accomplished by ultrafiltration with Amicon cells and PM-10 membranes. Potassium pantoate was prepared from pantoyl lactone (3). Thin layer chromatography was performed with cellulose plates.

**RESULTS**

To obtain information about the precursor of \( \beta \)-alanine in E. coli, experiments were performed in which cells (taken during exponential growth) were incubated with pantohydroc acid, glycine, and compounds suspected as precursors of \( \beta \)-alanine, and the amount of pantothenate synthesized was measured by microbiological assay. The results (Table I) suggest that aspartic acid is the precursor, since it was the only compound tested which could replace \( \beta \)-alanine for pantothenate synthesis.

**Confirmation of this suggestion was obtained with the observation that \( \beta \)-alanine (measured by microbiological assay) was produced by incubation of L-aspartate with extracts of E. coli. For subsequent studies, we used the more convenient radioactive assay in which the amount of radioactive product (\( \beta \)-alanine or \( CO_2 \), or both) produced from \( ^{14}C \)-labeled aspartate is determined. With crude extracts, 65 to 75% of \( ^{14}CO_2 \) released from DL-L-\( ^{14}C \) aspartate can be correlated with \( \beta \)-alanine production (Table II). The production of \( ^{14}CO_2 \) from L-\( ^{14}C \) aspartate in the presence of a crude extract is linear with time up to 75 min of incubation and with protein concentration up to 8 mg/reaction mixture.

**Purification of the Enzyme**

**Preparation of Crude Extract**—Freshly grown cells were used to prepare extracts, since storage of frozen cells resulted in loss of activity. Frozen cells (400 g, moist weight) were ruptured in a Hughes press, and the resulting frozen material was suspended in a liter of Buffer A (50 mM potassium phosphate, 5 mM EDTA, and 50 \( \mu \)M dithiothreitol, pH 7.0). After the material had thawed, the suspension was homogenized in a Waring Blender, and the material was incubated with DNase (50 \( \mu \)g) for 20 min at room temperature. The "crude extract" was obtained by removal of insoluble material by centrifugation at 24,000 \( \times \) g for 2 h.

**Heat Treatment**—The crude extract (993 ml) was warmed (in 200-ml portions) at 55°C and maintained at that temperature for 3 min, after which each portion was cooled rapidly to 4°C. The resulting precipitate was removed by centrifugation and discarded.

**Fractionation with Ammonium Sulfate**—The heat-treated extract (960 ml) was treated with solid ammonium sulfate at 4°C, to yield a fraction which precipitated between 40 and 60% saturation. The precipitate was dissolved in 150 ml of Buffer A, and the solution was dialyzed against two 6-liter portions (8 h each) of Buffer A.

**Fractionation on DEAE-Sephadex A-50**—The dialyzed ammonium sulfate fraction (180 ml, 11 g of protein) was applied to a column (8.1 x 40 cm) of DEAE-Sephadex A-50 that had been equilibrated with Buffer A. The column was developed by a linear gradient (0 to 0.3 M) of KCl, formed by placing 4 liters of Buffer A in the mixing chamber and 4 liters of the same buffer containing 0.3 M KCl in the reservoir. Fractions of 25 ml each were collected at a rate of 100 ml/h. A portion of the enzyme activity was eluted (Fractions 11 to 35) in the absence of KCl in the developing buffer, and a second much larger peak of activity (Fractions 275 to 390) appeared during the development in the presence of the KCl.

### Table I

<table>
<thead>
<tr>
<th>Substrate</th>
<th>CO(_2)</th>
<th>( \beta )-Alanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-L-[( ^{14} )C]aspartate</td>
<td>9.3</td>
<td>0</td>
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<tr>
<td>DL-[( ^{4} )C]aspartate</td>
<td>3.6</td>
<td>7.1</td>
</tr>
<tr>
<td>L-[( ^{14} )C]aspartate</td>
<td>10.2</td>
<td>5.9</td>
</tr>
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**Stimulation in resting cells of E. coli of the synthesis of pantothenic acid by aspartic acid**

In these experiments the general procedure was that used by Ortega and Brown (37) to measure the synthesis of nicotinic acid by resting cells of E. coli B. The reaction mixtures contained, per 2.0 ml total volume: 100 mM glucose, 1 mM pantoate (potassium salt), 0.0 ml of a washed cell suspension in 200 mM Tris-HCl buffer at pH 7.5 (dry weight of cells was 30 mg), and either 1 mM L-\( \beta \)-alanine or 1 mM putative precursor of \( \beta \)-alanine. Incubation was for 45 min at 37°C, after which the mixtures were boiled to lyse the cells. The soluble material was separated from cell debris by centrifugation, and, after a brief (20 min) incubation with alkaline phosphatase (4 mg) to convert any phosphopantothenate present to pantothenate, each extract was analyzed for pantothenate by microbiological assay with S. carlsbergensis 4228. In the results shown, the amount of endogenous pantothenate present in cells at zero time has been subtracted.

**Table II**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Radioactive product formed</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CO(_2)</td>
</tr>
<tr>
<td>DL-L-[( ^{14} )C]aspartate</td>
<td>9.3</td>
</tr>
<tr>
<td>DL-[( ^{4} )C]aspartate</td>
<td>3.6</td>
</tr>
<tr>
<td>L-[( ^{14} )C]aspartate</td>
<td>10.2</td>
</tr>
</tbody>
</table>
The fractions from the two peaks were combined (3100 ml), and the protein was precipitated by the addition to the solution of 1577 g of ammonium sulfate (to make a solution 80% saturated with the salt). The resulting precipitate was recovered by centrifugation and dissolved in Buffer A (40 ml). The resulting solution was dialyzed against two 6-liter portions (8 h each) of the same buffer.

Fractionation of QAE-Sephadex Q-50—The dialyzed solution (58 ml, 1.1 g of protein) from the previous step was subjected to fractionation on a column of QAE-Sephadex Q-50 (see Fig. 1). Two peaks of activity were evident; one (Peak I, 15% of the total activity) appeared in Fractions 21 to 36 (without the KCl), and the remainder of the activity appeared (Peak II) in Fractions 300 to 370. The fractions of Peak I were combined and concentrated to 6.6 ml. This enzyme activity was not fractionated further. Peak II fractions were combined, concentrated to 20 ml, and dialyzed (4°C) for 12 h against 6 liters of Buffer A.

Fractionation on Ultrogel AcA 44—The dialyzed solution (20 ml, 696 mg of protein) from the previous step was applied to a column (5.4 x 110 cm) of Ultrogel AcA 44 which had been equilibrated with Buffer A. The column was developed (4°C) with the same buffer, and fractions (11.2 ml each) were collected at a rate of 45 ml/h. Fractions (94 to 100) which contained enzyme activity were combined and concentrated to 8.0 ml, after which the solution was dialyzed against two 6-liter portions (8 h each) of Buffer B (50 mM potassium phosphate, 5 mM EDTA, and 50 μM dithiothreitol, pH 7.0).

Fractionation on Hydroxyapatite—The preparation (9.0 ml, 146 mg of protein) from the previous step was applied to a column (3.7 x 27 cm) of hydroxyapatite which had been equilibrated with Buffer B. The column was developed with the same buffer, and fractions of 6.6 ml each were collected at a rate of 50 ml/h. Fractions (9 to 27) that contained enzyme activity were combined and concentrated to 9.6 ml.

Preparative Scale Isoelectric Focusing—The preparation (9.6 ml, 23 mg of protein) from the previous step was subjected to isoelectric focusing on an LKB 8101 (110-ml capacity) column. The sucrose gradient was formed manually and contained 6% ampholytes (pH 3.5 to 5.0). After the pH gradient had formed (after 39½ hours at 400 V, 10°C), the column was emptied at a rate of 1.0 ml/min, and 1.0 ml fractions were collected. Fractions that contained enzyme activity were combined and used to form a second gradient without the addition of more ampholytes. The focusing procedure was repeated for 40.3 h at 450 V, after which the column was emptied at a rate of 0.5 ml/min. Fractions (1.0 ml each) containing activity were combined and filtered through an Ultrogel AcA column (5.4 x 110 cm, equilibrated and developed with Buffer A) to remove the ampholytes. Fractions (11.2 ml each) containing enzyme activity were combined and concentrated to 0.5 ml.

Preparative Gel Electrophoresis—Polyacrylamide gels (10% acrylamide, 1.4 x 12.6 cm) were prepared (31) with Tris/Na2-EDTA/boric acid, pH 8.3 (32), as the buffer in the electrode chambers. To the enzyme solution (0.5 ml, 4 mg of protein) from the previous step was added 0.1 ml of 0.05% bromphenol blue (dye marker), and 0.2 ml of the preparation was added to each of three gels. Electrophoresis was at 4°C at 10 mA/gel until the dye marker was 1.5 cm from the end of the gel. Each gel was cut into slices (0.5 cm thick), and the corresponding slices from the three gels were combined and washed with four 3-ml portions of Buffer A at 4°C over a 36-h period. The four washes from each set were combined, concentrated to 2.0 ml, and assayed for activity. Fractions of highest specific activity were combined and filtered through a column (1.2 x 118 cm) of Ultrogel AcA 54 (equilibrated and developed with Buffer A) to remove traces of acrylamide. Analyses of fractions (1.0 ml each) revealed a single peak of enzyme activity which coincided with a single protein peak. Fractions (67 to 85) containing the activity were combined and concentrated to 3.5 ml. This preparation, called the “purified enzyme,” can be stored for at least 6 months at 4°C without loss of activity, but activity is lost gradually if the material is stored frozen.

The summary of the purification procedure given in Table III indicates an apparent overall purification of 3800-fold. It seems likely that fractionation on the hydroxyapatite column removed an inhibitor, since the amount of activity recovered (3144 units) significantly exceeded the amount (2422 units) applied to the column.

### Chemical and Physical Properties of the Enzyme

Since analyses of the purified enzyme preparation by electrophoresis at pH 7.0 and 9.5 indicated the presence of a single protein band (Fig. 2) which coincided exactly with the location of the fractions that contained enzyme activity, the purified enzyme was stored frozen.

#### Table III

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Total activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Specific activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Relative specific activity&lt;sup&gt;a&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>units</td>
<td>units/mg</td>
<td></td>
</tr>
<tr>
<td>Crude extract</td>
<td>6700</td>
<td>0.17</td>
<td>1.0</td>
</tr>
<tr>
<td>Host-treated extract</td>
<td>6940</td>
<td>0.24</td>
<td>1.4</td>
</tr>
<tr>
<td>Ammonium sulfate fraction</td>
<td>6270</td>
<td>0.57</td>
<td>3.4</td>
</tr>
<tr>
<td>Eluate from DEAE-Sephadex</td>
<td>5920</td>
<td>5.6</td>
<td>33</td>
</tr>
<tr>
<td>Eluate from QAE-Sephadex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak I</td>
<td>680</td>
<td>23</td>
<td>137</td>
</tr>
<tr>
<td>Peak II</td>
<td>3970</td>
<td>5.7</td>
<td>34</td>
</tr>
<tr>
<td>Eluate from Ultrogel AcA44</td>
<td>2420</td>
<td>17</td>
<td>98</td>
</tr>
<tr>
<td>Eluate from hydroxyapatite</td>
<td>3140</td>
<td>140</td>
<td>800</td>
</tr>
<tr>
<td>Material from isoelectric focusing step</td>
<td>1380</td>
<td>350</td>
<td>2000</td>
</tr>
<tr>
<td>Material from preparative electrophoresis</td>
<td>710</td>
<td>650</td>
<td>3800</td>
</tr>
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</table>

<sup>a</sup> One unit of activity is the amount of enzyme needed for the production of 1 nmol of CO2/min at 42°C under standard assay conditions.

<sup>b</sup> Units of enzyme/mg of protein.
of enzyme activity on the gels, we conclude that the purified enzyme is a homogeneous protein.

The isoelectric point of the enzyme was determined to be pH 4.67, calculated as the average from seven separate determinations as described earlier in the purification section concerned with preparative isoelectric focusing. The molecular weight of the enzyme was estimated at 58,000 by a comparison of its rate of filtration through a column of Ultrogel AcA 54 with the rates of standard proteins (and their molecular weights) as follows: bacterial alkaline phosphatase (80,000), ovalbumin (45,000), chymotrypsinogen (25,000), myoglobin (17,000), RNase A (13,700), and cytochrome c (11,700). The accuracy of the estimations of the molecular weights of the proteins of Bands c and d depends on the assumption that migration remains a linear function of the log of the molecular weight all the way out to the position of the tracking dye on the 15% gels.

Additional experiments have indicated that heating the purified enzyme at 100°C in the absence of sodium dodecyl sulfate caused the partial transformation of Band a protein into Bands b, c, and d. The reason for this transformation remains unknown. One possibility is that the purified enzyme was contaminated with trace amounts of protease; however, this seems unlikely, since we have shown in other experiments that standard proteins, when incubated with the purified enzyme, are not degraded into smaller molecular weight units.

Identification of β-Alanine as Product

The results presented in Fig. 4 show that the use of L-[U-
Aspartate-α-decarboxylase

[14C]aspartate (Panel A) or DL-[14C]aspartate (Panel C) as substrate yielded a radioactive product which migrated as standard β-alanine, whereas, as expected, no such radioactive product was evident with DL-[1-14C]aspartate as substrate (Panel B). The data presented in Fig. 4 also indicate that the enzyme does not use D-aspartate as substrate, since the reaction with L-[1-14C]aspartate resulted in virtually complete conversion of the substrate to β-alanine (Panel A, Fig. 4), whereas when DL-[4-14C]aspartate was added only approximately half of the radioactive material appeared as β-alanine. Qualitative determinations (Table IV) indicate that the production of radioactive material with the characteristics of β-alanine approximately equaled the amount of radioactive CO₂ produced. These results clearly show that in the presence of the purified enzyme no product other than CO₂ and putative β-alanine is produced.

Confirmation that β-alanine is the product was obtained with the observations that, in the presence of the purified enzyme, L-aspartate (but not D-aspartate) was converted to a product which was active in replacing β-alanine for the growth of S. carlbergensis (as determined as described under “Materials and Methods”), and finally we determined that the radioactive compound produced from L-[1-14C]aspartate is ninhydrin-positive and behaves as authentic β-alanine when subjected to thin layer chromatography in six solvent systems. The solvent systems used with the RF values (development was in the ascending fashion) for standard β-alanine were as follows: phenol/NH₄OH (200:1, by volume), RF = 0.71; phenol/ethanol/H₂O/NH₄OH (150:40:10:1, by volume), RF = 0.43; 1-butanol/pyridine/H₂O (60:60:60, by volume), RF = 0.26; methanol/H₂O/pyridine (160:40:8, by volume), RF = 0.90; ethanol/H₂O/NH₄OH (80:10:10, by volume), RF = 0.57; and 1-butanol/acetic acid/H₂O (12:3:5, by volume), RF = 0.53.

Table IV

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Radioactive product formed</th>
<th>CO₂</th>
<th>β-Alanine</th>
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<tbody>
<tr>
<td>L-[1-14C]Aspartate</td>
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<td>21.6</td>
<td>23.2</td>
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<tr>
<td>DL-[1-14C]Aspartate</td>
<td></td>
<td>21.6</td>
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<tr>
<td>DL-[4-14C]Aspartate</td>
<td></td>
<td>0</td>
<td>24.4</td>
</tr>
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</table>

Catalytic Properties of the Enzyme

From an Edie-Hofstee plot, we have calculated the Kₘ for L-aspartate to be 0.16 mM, determined with the use of the purified enzyme. The following compounds (with the inhibition constant for each) were found to be competitive inhibitors: L-glutamate, 0.76 mM; succinate, 0.73 mM; oxaloacetate, 0.81 mM; L-serine, 0.73 mM; L-cysteic acid, 0.08 mM; β-hydroxy-DL-aspartate, 0.13 mM; and D-serine, 0.16 mM. D-Aspartate is neither a substrate nor an inhibitor. The following compounds have no effect as inhibitors at concentrations as high as 0.25 mM (at a concentration of 1 mM for the substrate, L-aspartate): β-alanine, acetyl coenzyme A, coenzyme A, and D-panthothenic acid. Thus, there is no evidence for any regulation of the activity of the enzyme by feedback inhibition by metabolic end products.

The pH optimum for the action of the enzyme is rather broad, between pH 6.5 and 7.5, but the enzyme functions half-maximally or greater between pH 5.3 and pH 8.6. The enzyme also acts over a relatively wide range of temperature, with half-maximal activity at 26°C and 78°C and an optimum at 55°C.

The activity of the enzyme is unaffected by monovalent cations such as Li⁺, Na⁺, NH₄⁺, and K⁺ at concentrations of 10 mM, however, KCl at 200 mM inhibited by 59%. The activity was unaffected by the following divalent cations at concentrations of 10 mM: Mn²⁺, Mg²⁺, Ca²⁺, Fe²⁺, and Co²⁺. Activity was inhibited by 88% by 10 mM CuCl₂.

Properties of Enzyme in Peak I from QAE-Sephadex Column

Rechromatography of Peak I material (see Fig. 1) on either DEAE-Sephadex or QAE-Sephadex showed that the active material was eluted from the columns again in a single peak in the void volume with no KCl in the developing buffer, an indication that the appearance of two peaks in the original fractionation scheme could not be explained as an overloading of the columns. The molecular weight (250,000) is much higher than that of the purified enzyme derived from Peak II material in Fig. 1. The temperature and pH optima of the two enzyme preparations are identical, but the Km for aspartate for the Peak I material (0.30 mM) is somewhat higher than that (0.16 mM) for the purified enzyme.

One rather curious and as yet unexplained observation is that a significantly lower proportion of the total enzyme activity in extracts of cells grown on a minimal medium was present as this high molecular weight form than that found in extracts of cells grown on a rich medium.

Identification of the Prosthetic Group as a Pyruvoyl Group

We found that the activity of the purified enzyme was completely destroyed by incubation for 10 min at 42°C with either 5 mM hydroxylamine, 5 mM phenylhydrazine, or 1 mM sodium borohydride. This strongly suggests that the decarboxylase contains a carbonyl-containing compound as a prosthetic group. The logical candidate is pyridoxal phosphate, since this coenzyme functions as a prosthetic group for a variety of other amino acid decarboxylases. However, no vitamin B₆ compound could be found when a hydrolysate of the purified enzyme was analyzed for vitamin B₆ by microbiological assay according to the procedure of Snell (39). Furthermore, the ultraviolet absorption spectrum of the purified enzyme (see Fig. 5) exhibited no peak of absorption other than that at 280 nm. From these observations we concluded that no pyridoxal phosphate is present.

Reaction of Enzyme with [U-14C]Phenylhydrazine—Confirmation that the enzyme contains a carbonyl compound was obtained with the finding that phenylhydrazine binds to the enzyme irreversibly and inactivates it. For this purpose, the purified enzyme (0.97 mg of protein in 1.0 ml of Buffer A) was incubated with [U-14C]phenylhydrazine (2.7 mM, 5.5 mCi per mmol) for 2½ hours at 42°C. The excess phenylhydrazine was removed by filtration through a column (1.8 X 36 cm) of Sephadex G-25, equilibrated, and developed with 50 mM ammonium bicarbonate. Fractions of 1.2 ml each were collected at a rate of 50 ml/h. A single protein peak was eluted (Fractions 31 to 39) which coincided exactly with a radioactive peak. These fractions were combined and reduced in volume to 1.2 ml. To ensure that all of the radioactivity consisted of phenylhydrazine covalently bound to the protein, a portion of the treated enzyme was dialyzed against several changes of
Buffer A. More than 90% of the radioactivity remained with the protein fraction. Another portion of the treated enzyme was mixed with 10% trichloroacetic acid in the presence of bovine serum albumin to ensure complete precipitation of the protein, 90% of the radioactivity was precipitated with the protein. Finally, a portion of the ¹⁴C-labeled, enzymatically inactive protein was mixed with active enzyme, and the mixture was subjected to electrophoresis on polyacrylamide gel as described earlier in this paper. Analyses of three identically prepared gels for protein, enzymatic activity, and radioactivity revealed that all were present in the same area of the gels.

In Fig. 5 are presented the absorption spectra of the purified enzyme before and after treatment with phenylhydrazine. The spectrum of the treated enzyme contained a shoulder at 320 nm not present in that of the untreated enzyme. The phenylhydrazones of pyruvate and α-ketobutyrate, prepared by the method of Riley and Snell (40), were found to absorb maximally at 312 nm. Thus, it appeared possible that the shoulder observed at 320 nm could be the result of the formation of a phenylhydrazone with either a pyruvate or α-ketobutyrate residue bound covalently to the enzyme. From the amount of ¹⁴C incorporated and the specific radioactivity of the [U-¹⁴C]phenylhydrazine used, we calculated that 1.1 mol of phenylhydrazine was formed/mol (molecular weight, 58,000) of enzyme. This suggests that there is 1 mol of carbonyl-containing prosthetic group/mol of enzyme.

Treatment of Enzyme with Sodium [³H]Borohydride—In order to obtain evidence about the nature of the carbonyl-containing component of the enzyme, the purified enzyme (1.5 mg of protein in 0.1 ml of Buffer A) was subjected to reduction by mixing it with 0.1 ml of 230 mCi/ml sodium [³H]borohydride (270 mCi/mmol) in 0.1 M sodium borohydride (pH 9.5) and allowing the mixture to stand at 42°C for 1 h. The solution was then dialyzed against two 1-liter portions of 50 mM ammonium bicarbonate (15 h each) to remove most of the excess tritiated material not bound to the protein. After dialysis, the protein solution was applied to a column (2 × 35 cm) of Sephadex G-25 which had been equilibrated with the dialysis solution, and the column was developed with the same solution. Fractions (1.0 ml each) were collected at a rate of 50 ml/h. A single protein peak was evident (Fractions 42 to 62), and the peak coincided exactly with a single radioactive peak. These fractions were combined and reduced in volume to approximately 2 ml.

Identification of Lactate as Tritiated Product—The concentrated, tritiated protein solution was evaporated to dryness under vacuum, and the residue was taken up in constant-boiling HCl (1.0 ml). This material was then heated at 104°C for 24 h to hydrolyze the protein. The resulting hydrolysate was evaporated to dryness under vacuum, and the residue was dissolved in 0.5 ml of 0.2 M pyridine/acetate buffer, pH 3.1. This solution was applied to a column (1.4 × 10.6 cm) of AG 50W-X4 (100 to 200 mesh, hydrogen form) that had been equilibrated with 0.2 M pyridine/acetate buffer (pH 3.1), and the column was developed with the same buffer. Fractions of 1.0 ml each were collected at a rate of 30 ml/h. The radioactivity (90% of the amount applied to the column) appeared as a single peak. The fractions constituting the peak were combined and evaporated to dryness. The residue was dissolved in 0.5 ml of water, and a portion (0.1 ml) was mixed with DL-[U-¹⁴C]lactate to give a solution containing (per 0.01 ml) 20,000 cpm of tritium and 700 cpm of ¹⁴C-labeled lactate. This mixture was subjected (0.01-ml amounts) to thin layer chromatography with five different solvent systems. In each system the material labeled with tritium migrated exactly as the standard ¹⁴C-labeled lactate. These solvent systems and the Rf values (development was by the ascending fashion) of lactate were as follows: I, 0.5 M ammonium acetate/acetate (1:3, by volume) (41), Rf = 0.46; II, 1-propanol/NH₄OH (8:2, by volume) (41), Rf = 0.62; III, 1-butanol/acetic acid/water (12:5, by volume) (42), Rf = 0.81; IV, 1-propanol/NH₄OH (4:1, by volume) (42), Rf = 0.43; and V, ethanol/1 M ammonium acetate (7:3, by volume) (40), Rf = 0.79. The coincident migration of the tritiated material with the standard ¹⁴C-labeled lactate is illustrated by three representative examples presented in Fig. 6, which show that on the developed thin layer plates the ratios of tritium to ¹⁴C across the areas of the radioactive zones of migration were constant.

Confirmation that the tritiated material was lactate was obtained with the observation that tritium was transferred from the tritiated product to NAD⁺ (to produce tritiated NADH) in the presence of lactate dehydrogenase. For this purpose, reaction mixtures were prepared as described in the legend of Fig. 7 to contain as substrate NAD⁺ and either authentic tritiated lactate (as a positive control) or the tritiated product isolated from the enzyme hydrolysate. The incubated reaction mixtures were then subjected to paper chromatography to separate the NADH from the residual lactate. The details of these procedures are given in the legend of Fig. 7. The results clearly show that lactate dehydrogenase catalyzed the production of tritiated NADH from the radioactive product obtained from the enzyme hydrolysate (Fig. 7B). The results presented in Fig. 7A show that under the same conditions radioactive NADH was produced in a control reaction mixture which contained standard tritiated lactate as substrate. For each reaction mixture (shown in Fig. 7, A and B) a certain amount of radioactive material migrated slightly more slowly than the radioactive NADH. Riley and Snell (40) also observed this kind of result in similar analyses, and they concluded that the material was radioactive NAD⁺ formed by oxidation of the [³H]NADH during chromatography. Although we have not identified this material as NAD⁺, it is likely that our results can also be explained in a similar way.

The production of tritiated NADH from the product in the presence of lactate dehydrogenase and the results of the thin layer chromatography analyses lead to the conclusion that the radioactive product isolated from the hydrolysate of the purified enzyme was lactate, a fact which indicates that the carbonyl-containing prosthetic group present in the enzyme is a pyruvoyl residue.

From the specific activity of the tritiated sodium borohydride and the determination of the radioactivity of the triti-
Aspartate-α-decarboxylase

FIG. 6. The coincident migration of tritium-labeled product (obtained from the hydrolysate of purified enzyme treated with tritiated borohydride) and standard [14C]-labeled lactate. The mixture of material containing the tritiated product and standard [14C]-labeled lactate is described in the text. This mixture was subjected to thin layer chromatography with Solvents I (Panel A), II (Panel B), and III (Panel C) as described in the text. Each developed plate was dried and cut into sections 0.5 cm wide. Each section was analyzed for radioactivity (both tritium and [14C]) in a scintillation counter.

To obtain information bearing on the question as to whether the enzyme functions biosynthetically for the formation of β-alanine used for the synthesis of pantothenic acid, the amount of enzyme activity present in a β-alanine (or pantothenate)-requiring mutant (E. coli 99-2) was compared with the amount present in the parent strain (E. coli W) from which the mutant was derived and with the amount observed in a revertant of the mutant. The specific activities (in enzyme units/mg of protein) of crude extracts of the wild type strain, the mutant, and the revertant were 0.46, 0.04, and 2.2, respectively. The specific activity of a crude extract of E. coli B, from which the enzyme was purified, is usually about 0.2. Obviously E. coli W contains somewhat larger amounts of enzyme activity than does E. coli B, and the revertant contains much larger amounts for reasons that remain unexplained.

The results presented in Fig. 8 show that both the higher molecular weight (smaller peak of activity) and the lower molecular weight forms of the decarboxylase are present in E. coli W and the revertant, but that both are virtually missing from E. coli 99-2, the β-alanine-requiring mutant.

FIG. 7. Reaction of tritiated product (obtained from hydrolysate of purified enzyme treated with tritiated borohydride) with NAD+ in the presence of lactate dehydrogenase. Reaction mixtures were prepared as recommended by Hohorst (43) to contain 71 units of lactate dehydrogenase and either 2 x 10^6 cpm of standard [3H]lactate (Panel A) prepared as described by George and Phillips (41), or 2 x 10^6 cpm of tritiated product from the enzyme hydrolysate (Panel B). Incubation was allowed to proceed at room temperature until the maximal amount of NAD+ had been converted to NADH (followed by observation of the increase in absorbance at 340 nm in a spectrophotometer). When no further increase in absorbance was observed, nonradioactive carrier NADH was added to each reaction mixture, and 0.5-ml portions were spotted on 3-cm-wide strips of Whatman No. 3MM paper. The chromatograms were developed (approximately 16 h), in the descending fashion, with ethanol/1M ammonium acetate (7:3, by volume). In Panel A, the positions of the radioactive materials were located by scanning the strips with a Packard model 7200 radiochromatogram scanner. In Panel B, the positions of the radioactive materials were determined by cutting the paper strip into sections and determining the radioactivity in each section in a scintillation counter. The cross-hatched areas indicate the zones of migration of the blue-fluorescent standard NADH.

FIG. 8. Analyses of crude extracts of E. coli W, β-alanine-requiring mutant (99-2) of E. coli W, and a revertant of the mutant for the high molecular weight and low molecular weight forms of aspartate-α-decarboxylase. Cells were grown on a rich medium (see "Materials and Methods"). Extracts were made and subjected to fractionation through the DEAE-Sephadex step (the heat treatment was omitted) by the general procedures described in an earlier section of this paper. The amounts of protein subjected to chromatography on individual columns of DEAE-Sephadex A-50 (each column was 1.4 x 11 cm) were 73, 63, and 36 mg for materials derived from E. coli W, E. coli mutant 99-2, and the revertant, respectively. The elution patterns are shown as (○) for material prepared from E. coli W, (□) for material prepared from the mutant, and (△) for material prepared from the revertant.
**DISCUSSION**

The most compelling evidence that the aspartate-α-decarboxylase purified by us is the enzyme responsible for the formation of β-alanine needed for the biosynthesis of pantothenic acid is that this enzyme is missing in the mutant (99-2) of *E. coli* that requires either β-alanine or pantothenate as a nutritional factor. Other evidence which supports this conclusion is that the action of the decarboxylase is strongly inhibited by d-serine, β-hydroxy-D,L-aspartic acid, and L-cysteic acid, three compounds that are known to interfere *in vivo* with the synthesis of pantothenic acid in bacteria (see the introduction). The decarboxylase we have purified is clearly different from the one studied by Nakano and Kitaoka (27), since the synthesis of their enzyme was induced by growth of *E. coli* at acidic pH values with large quantities of aspartate in the medium as a nitrogen source. We have found that growth under these conditions does not increase the amount of our decarboxylase over that observed during growth at neutral pH on an aspartate-free medium. It seems likely that the enzyme studied by Nakano and Kitaoka is a catabolic enzyme. A number of such inducible catabolic amino acid decarboxylases have been described in bacteria, including *E. coli* (44).

The significance of the occurrence in *E. coli* of the high molecular weight form of aspartate-α-decarboxylase (Peak I activity in Fig. 1) is not clear. Since it represents only a small amount of the total decarboxylase activity of extracts, we have not yet attempted to purify enough of this form of the enzyme to allow the same kinds of analyses that were done with the 58,000-dalton variety. For example, the Peak I enzyme has not been analyzed for pyruvate as a prosthetic group, although certain other properties of the enzyme are similar to those of the purified enzyme.

One of the most interesting results of our investigations is the observation that *E. coli* aspartate-α-decarboxylase contains a pyruvyl residue as a prosthetic group rather than pyridoxal phosphate, which is the coenzyme present in most other bacterial amino acid decarboxylases. This enzyme thus joins a growing list of enzymes that contain covalently bound formation of β-alanine needed for the biosynthesis of pantothenic acid. The pyruvyl group participates in catalysis, although this seems very likely to be true because of the marked sensitivity of the enzyme to reagents such as phenylhydrazine.

As yet no definitive explanation is available for the formation of the three low molecular weight polypeptides when the purified enzyme is heated with sodium dodecyl sulfate. Several possibilities present themselves. One is that the enzyme consists of three subunits that are so firmly bound together that relatively drastic denaturing conditions are needed to dissociate the enzyme. If this is true, one might expect that the catalytically active (58,000-dalton) form of the enzyme consists of two each of the three polypeptide chains of molecular weights 11,800, 9,800, and 6,400, since the molecular weights of these three add up to approximately half of that of the undenatured protein.

Another possible explanation is that, during preparation of the extracts and purification of the enzyme, proteolysis of the enzyme occurs at specific susceptible peptide bonds, but that the secondary and tertiary forces holding the protein together are strong enough to keep the protein in its catalytically active state. One final possibility is that treatment under the denaturing conditions causes the cleavage of particularly susceptible peptide bonds. Seto (50) has suggested a similar explanation for the observation that a proline reductase from *C. sticklandii* can be cleaved under relatively mild conditions.

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Purification and properties of L-Aspartate-alpha-decarboxylase, an enzyme that catalyzes the formation of beta-alanine in Escherichia coli.

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