Decarboxylation of γ-Hydroxyglutamate by Glutamate Decarboxylase of *Escherichia coli* (ATCC 11246)*

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ABSTRACT: Glutamate decarboxylase has been purified approximately 15-fold from extracts of *Escherichia coli* (ATCC 11246). This enzyme preparation catalyzes decarboxylation of the α-carboxyl group of *threo*-γ-hydroxy-L-glutamate with the formation of carbon dioxide and α-hydroxy-γ-aminobutyrate. The following observations strongly suggest that γ-hydroxyglutamate and glutamate are decarboxylated by the same enzyme. (1) At all stages of purification, the ratios of specific activity of γ-hydroxyglutamate to glutamate are essentially constant and the percentage of units recovered is the same whether calculated for either amino acid; (2) decarboxylase activity for both substrates changes at the same rate and in the same manner during controlled heat treatment; (3) hydroxylamine and α-methylglutaric acid are competitive inhibitors for the decarboxylation of either glutamate or γ-hydroxyglutamate and K_I values determined for a given inhibitor are the same for either amino acid; and (4) the partially purified decarboxylase has the same pH–activity curve for both substrates. Only the *threo* isomer of γ-hydroxy-L-glutamate serves as substrate for the enzyme; the *erythro*-L isomer and the corresponding D diastereoisomers are enzymatically inactive. Of some 28 substituted forms of glutamic acid or related compounds tested, only β-hydroxyglutamate, γ-methyleneglutamate, γ-hydroxyglutamate, γ-benzylglutamate, γ-methylglutamate, and the γ-methyl ester of glutamic acid are active.

The enzymatic decarboxylation of L-glutamic acid is well known; the product formed, γ-aminobutyrate, appears to play a unique role in tissues of the central nervous system of mammals. There is also a specific decarboxylase for β-hydroxyglutamate; independent papers confirm the presence of this enzyme in bacteria (Umbreit and Heneage, 1953) and in brain (Hisada and Nakashima, 1960).

γ-Hydroxyglutamate is a normal intermediate in the mammalian metabolism of L-hydroxyproline (Adams and Goldstone, 1960a) and may possibly be involved in glutamate metabolism of *Acetobacter suboxydans* (Sekizawa *et al.*, 1966). It is also present in large amounts as the free amino acid in certain plants (Virtanen and Hietala, 1955a). Preliminary reports have indicated that γ-hydroxyglutamate is also decarboxylated by mammals (Bouthillier and Binette, 1961) and bacteria (Virtanen and Hietala, 1955b). No detailed study of this enzymatic reaction, however, has appeared. In addition, the question of whether or not a specific enzyme catalyzes the reaction remains unsettled. Much of the literature pertaining to this and related reactions would seem to convey the suggestion that a unique decarboxylase may be required. For example, Kuratomi *et al.* (1963) reported that rat liver homogenates catalyze a vigorous decarboxylation of γ-hydroxyglutamate; mammalian liver is known to contain very little or no glutamate decarboxylase activity. In observing the decarboxylation of glutamate, β-hydroxyglutamate, and γ-hydroxyglutamate by a mutant of *Escherichia coli*, Virtanen and Hietala (1955b) suggested that different enzymes may be involved. The identity of the enzyme in brain homogenates that catalyzes γ-hydroxyglutamate decarboxylation (Bouthillier and Binette, 1961) was not examined.

We have begun a detailed study of the glutamate decarboxylase of *E. coli* ATCC 11246 (the source of this enzyme sold commercially) since: (a) a simple procedure for preparing purified enzyme from this starting material has not been reported nor are the properties of the purified enzyme known; (b) the characteristics of the enzyme obtained from this source have not been compared or contrasted with those reported for the glutamate decarboxylase purified from *E. coli* strain 26 (Shukuya and Schwert, 1960); (c) γ-hydroxyglutamate and other naturally occurring glutamic acid analogs have not been tested as substrates for this purified enzyme; and (d) the possible interaction of substrate analogs with glutamate decarboxylase might yield vital information regarding the nature of the active site.

This paper presents evidence that the α-carboxyl group of γ-hydroxyglutamate is decarboxylated by glutamate decarboxylase; α-hydroxy-γ-aminobutyrate is the new product. The properties of the reaction and of the product formed are listed. A preliminary report of portions of this study appeared previously (Dekker and Homola, 1964).

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Experimental Procedures

Materials

threo-γ-Hydroxy-DL-glutamic acid was synthesized and characterized as described before (Dekker and Maitra, 1962); *erythro*-γ-hydroxy-DL-glutamic acid was obtained from the supernatant fluid remaining after the *threo* racemate had been precipitated. The *threo*-L isomer of γ-hydroxyglutamic acid was isolated from *Phlox decussata* (Dekker, 1962) or purchased from Calbiochem, Inc.; the *eythro*-L isomer was formed enzymatically from hydroxy-L-proline by the procedure of Adams and Goldstone (1960b). γ-Hydroxy[2-^{14}C]glutamic acid was prepared from [2-^{14}C]diethylacetamidomalonate according to the procedure of Benoiton and Bouthillier (1956). γ-Hydroxy-γ-methylglutamic acid was synthesized as outlined earlier (Maitra and Dekker, 1964). The method of Touster and Carter (1951) was followed to make β,γ-dihydroxyglutamic acid. The two stereoisomers of 2-keto-4-hydroxyglutaric acid were obtained by reaction of either diastereoisomer of γ-hydroxy-L-glutamic acid with pyridoxal and Cu^{2+} at 100°, as previously described (Maitra and Dekker, 1963). α-Hydroxy-γ-aminobutyric acid, a generous gift from Dr. A. Mori (Department of Neurological Surgery, Osaka University Medical School, Osaka, Japan), was purified to homogeneity by paper chromatography. This same compound was also prepared chemically in our laboratories by the following steps: reaction of α,γ-diaminobutyric acid with $CuCO_3$ to yield the crystalline copper complex; protection of the γ-amino group by reaction with carbobenzoxy chloride; decomposition of the copper complex with H_2S and removal of the copper sulfide by filtration; conversion of the α-amino group to a hydroxyl group by reaction with HONO at 0°; and removal of the carbobenzoxy group by hydrogenation in the presence of palladium on charcoal. The desired product was isolated and purified by adsorption and elution from columns of Dowex 50 (H^+) resin. The prepared amino acid was shown to be homogeneous and identical in mobility with the sample of Dr. Mori by paper chromatography in a variety of solvent systems. Gift samples of the following compounds are gratefully acknowledged: γ-hydroxy[5-^{14}C]glutamic acid from Dr. L. P. Bouthillier; *erythro*-β-hydroxy-DL-glutamic acid from Dr. S. Hisada; γ-methylene-DL-glutamic acid, γ-methyl-DL-glutamic acid, α,γ-diaminoglutaric acid, γ-ethyl-DL-glutamic acid, and β-methyl-DL-glutamic acid from Dr. W. A. Zygmunt (Mead Johnson Research Center). α-Methylglutaric acid was a product of K & K Laboratories, Inc. *threo*-β-Hydroxy-DL-glutamic acid was purchased from Fluka and the two racemates of *erythro*- and *threo*-β-hydroxyaspartic acid from Calbiochem. α-Hydroxy-L-glutaric acid was obtained from Cyclo Chemical Corp. Acetone powders of calf, rabbit, and pigeon brain were purchased from Nutritional Biochemicals Corp.; fresh animal tissues were obtained from local abattoirs.

An acetone powder of *E. coli* (ATCC 11246), purchased from Worthington Biochemicals Corp., served as starting material for purifying glutamate decarboxylase. All other compounds and materials not listed were commercial products.

Methods

Protein was usually measured by the method of Lowry *et al.* (1951) with crystalline bovine albumin as a standard; in crude homogenates of animal tissues, it was measured spectrophotometrically with a correction for nucleic acid content (Warburg and Christian, 1941). Quantitative ninhydrin determinations were made according to the procedure of Rosen (1957). Acidic amino acids were neutralized before use in enzymatic tests by careful addition of KOH solution. Absorbancy measurements were made at room temperature with a Beckman DU spectrophotometer. A thin-window gas-flow counter (Nuclear-Chicago Corporation) was used for assaying radioactive samples. Enzymatic liberation of carbon dioxide was followed with a conventional manometric Warburg apparatus (Precision Scientific Co.).

Enzyme Assay. Enzyme activity was determined manometrically by measuring the rate of carbon dioxide evolution. Unless indicated otherwise, the main compartment of the Warburg vessels contained 600 μmoles of sodium acetate buffer (pH 5.0), 300 μmoles of NaCl, either 25 μmoles of L-glutamate or 100 μmoles of *threo*-γ-hydroxy-DL-glutamate, and any other desired additions. The substrate was generally placed in the side arm; the volumes of the two compartments were adjusted with distilled, deionized water so that the final volume after mixing equalled 3.0 ml. The flasks and contents were shaken for 15 min at 37° before they were closed. After the reaction vessels were shaken for another 15 min at 37°, the substrate solution was mixed with the contents of the main compartment, and carbon dioxide evolution was recorded at 5-min intervals for a period of 30 min with L-glutamate as substrate and for 60 min with γ-HG.[1] Specific activity is expressed in μl of carbon dioxide evolved/10 min per mg of protein with L-glutamate as substrate, or in μl of carbon dioxide liberated/60 min per 10 mg of protein when γ-HG served as substrate. Under these assay conditions and within defined ranges, the rate of carbon dioxide evolution is proportional to the amount of protein added and to the length of time of incubation.

Results

Decarboxylation of γ-HG by Preparations of Mammalian Tissues. Homogenates of fresh mouse, rat, and beef brain as well as extracts of calf, rabbit, and pigeon brain acetone powders showed no significant decarboxylation of L-glutamate by manometric assay. Using a more sensitive technique, however, we found that homogenates of fresh mouse, rat, and beef brain catalyzed a decarboxylation of [2-^{14}C]γ-HG (the new product formed was detected by radioautography, as described later). All attempts to demonstrate decarboxylation of γ-HG by rat liver preparations, as reported by Kuratomi

[1] Abbreviation used: γ-HG, γ-hydroxyglutamate.

TABLE I: Purification of Glutamate Decarboxylase from *E. coli* ATCC 11246.[a]

	Fraction	Protein (mg/ml)	Sp Act.[b]	Total Units	Recov (%)
I	Crude extract	28.0	575	483,000	100
II	Supernatant after protamine sulfate addition	18.0	767	476,307	99
III	$(NH_4)_2SO_4$ precipitate (0–75%)	10.8	1148	216,972	45
IV	$(NH_4)_2SO_4$ precipitate (30–65%)	10.0	2000	184,000	38
V	$(NH_4)_2SO_4$ precipitate (35–55%)	14.6	3370	131,430	27
VI	Heated 45°, 1 hr	5.8	8800	105,600	22

[a] The activity of every fraction was measured with both L-glutamate and *threo*-DL-γ-HG as substrates; the specific activity values listed correspond to those obtained with L-glutamate. [b] Definition of specific activity: μl of CO_2/mg of protein per 10 min.

et al. (1963), failed. For this purpose, manometric assays were run as well as tests with [2-^{14}C]γ-HG followed by radioautography of two-dimensional chromatograms. Liver preparations tested included whole homogenates prepared by a variety of methods, the supernatant fluid of homogenates centrifuged for 1 hr at 30,000*g*, dialyzed supernatant fluid, or supernatant fluid passed over a column of Sephadex G-25; assays were run both anaerobically (under prepurified nitrogen gas) and aerobically; *erythro*-DL-γ-HG and *threo*-L-γ-HG were tested as substrates. High levels of radioactive γ-HG were used in these experiments with liver preparations and X-ray films were exposed to the two-dimensional chromatograms for periods up to 2 weeks. The only amino acids detected containing significant radioactivity were alanine and glycine; these products are formed from γ-HG in the liver by well established routes (Dekker and Maitra, 1962). Other compounds, known to be intermediates in the catabolism of γ-HG by liver enzymes (Maitra and Dekker, 1963), were also not decarboxylated by liver preparations; these compounds included L- and D-2-keto-4-hydroxyglutarate, sodium pyruvate, and sodium glyoxylate.

Purification of L-*Glutamate Decarboxylase from E. coli* (*ATCC 11246*). All operations were carried out between 0 and 4° unless otherwise stated.

Step 1. E. coli (ATCC 11246) acetone powder was suspended in water (1 g of powder/15–20 ml) and the mixture was exposed to sonic oscillation for two separate 1-min intervals (top power, Branson Sonifier Model LS-75). Insoluble material was removed by centrifugation at 30,000*g* for 1 hr.

Step 2. A 2% solution of protamine sulfate (pH 6.0) was added dropwise with constant stirring to the supernatant fluid (30 ml) from step 1; 0.15 mg of protamine was added/mg of protein in the extract. The suspension was stirred for an additional 20 min and the precipitate obtained by centrifugation was discarded.

Step 3. Crystalline ammonium sulfate was added in small portions with stirring to the supernatant solution (34 ml) until the salt concentration was 75% of saturation (520 g/l.). The mixture was stirred an additional 20 min. The protein precipitate obtained by centrifuging for 30 min at 30,000*g* was dissolved in 30 ml of cold 0.01 M sodium acetate buffer (pH 5.0) and dialyzed for 1.25 hr against 1 l. of the same acetate buffer.

Step 4. The resulting dialyzed solution was further fractionated by the addition of crystalline ammonium sulfate, as described above. The protein precipitate obtained between 30 (172 g/l.) and 65% (418 g/l.) of saturation was collected by centrifugation, dissolved in 12 ml of 0.01 M sodium acetate buffer (pH 5.0), and dialyzed as in step 3.

Step 5. A final fractionation with ammonium sulfate was carried out as before; the active protein fraction was precipitated between 35 (203 g/l.) and 55% (338 g/l.) of saturation. This precipitate, after being removed by centrifugation, was dissolved in 5.0 ml of cold 0.01 M sodium acetate buffer (pH 5.0).

Step 6. Portions (1 ml each) of the protein solution from step 5 were heated in loosely stoppered, 12-ml centrifuge tubes for 1 hr in a 45° water bath. The suspensions were then immediately cooled in ice. The precipitate of denatured protein was removed by centrifugation and discarded. At this stage, the enzyme solution has a bright yellow color and loses very little activity when stored at 4° for 2 weeks. Addition of pyridoxal 5′-phosphate to the fractions obtained at each stage of purification caused only a very slight increase in specific activity. The course of purification of the enzyme is summarized in Table I.

Comparative Activities of Decarboxylase Preparations toward L-*Glutamate and* γ-*HG.* In order to ascertain whether glutamate decarboxylase obtained from *E. coli* extracts also utilizes γ-HG as substrate, we determined the specific activities of the protein fractions obtained in purifying the decarboxylase with both L-glutamate and γ-HG as substrate (Table II). Although the final enzyme sample obtained in this instance had a specific activity value significantly lower than that normally achieved in the purification procedure, the protein fractions prepared at each step were suitable for the purpose in mind. As can be seen in Table II, L-glutamate is by far the more active substrate, but a measurable reaction is clearly

TABLE II: Activity of Decarboxylase Fractions with L-Glutamate and γ-Hydroxyglutamate.[a]

Fraction	Recov of Units (%)		Sp Act.[b]		Ratio of Sp Act. (*h*/*g*)
	Glutamate	γ-HG	Glutamate (*g*)	γ-HG (*h*)	
I	100	100	504	428	0.85
II	87	86	703	591	0.84
III	83	84	848	738	0.87
IV	60	69	1510	1470	0.97
V	54	54	3000	2550	0.85
VI	27	28	5600	4850	0.87

[a] The activity of every fraction was measured with both L-glutamate and *threo*-DL-γ-HG as substrates. The fraction numbers refer to those of Table I. [b] Definition of specific activity values: μl of CO_2/mg of protein per 10 min for L-glutamate and μl of CO_2/10 mg of protein per 60 min for *threo*-DL-γ-HG.

discernible with γ-HG (column *h*) when higher levels of protein and longer incubation times are used. In spite of this difference in reactivity, the ratios of specific activities for the two amino acids were found to be essentially constant. Also, the percentage of units recovered was the same whether calculated for either amino acid. In addition, fraction VI (Table I) was adsorbed to and eluted from a column of calcium phosphate gel–cellulose. Under the conditions used, decarboxylase activity was eluted as an extremely broad peak with no significant further purification accomplished. However, when a number of tubes in this elution series was analyzed for decarboxylase activity with γ-HG and glutamate as substrates, the ratios of specific activity were constant throughout.

Heat Denaturation Studies. An enzyme preparation (fraction V, Table I) was subjected to heat treatment at 50°, at which temperature decarboxylase activity is slowly destroyed over a period of 2–3 hr. For this purpose, 1-ml portions of the enzyme solution were dispensed into each of several tubes and heated in a constant temperature water bath. At five different time intervals (30–180 min) a tube was removed and immediately cooled in ice, and the denatured protein was removed by centrifugation. Enzymatic activity was measured after each period of heat treatment and the ratio of specific activity was determined, using L-glutamate and γ-HG separately as substrate. The ratios of specific activities (γ-HG: L-glutamate) again remained constant; a value of 0.87 for the initial, untreated enzyme preparation varied within the range of 0.85–0.93 throughout this heat denaturation process. The enzyme is completely inactivated by heating at 60° for 30 min.

Effect of pH on Decarboxylase Activity. The decarboxylase is active within the pH range of 4.5–5.8 with maximal activity at about 5.3. The same pH–activity curve is obtained using either L-glutamate or γ-HG as substrate (Figure 1).

Inhibition Studies. Both hydroxylamine and α-methylglutaric acid act as inhibitors of L-glutamate decarboxylation (Taylor and Gale, 1945; Roberts, 1953). These compounds also strongly inhibit the decarboxylation of γ-HG in a competitive manner. The dissociation constants of the enzyme inhibitor complexes (K_I's) were determined with each of the substrates, L-glutamate and γ-HG. Values were determined by Dixon (1953) plots for competitive inhibition. The values found for hydroxylamine were 1.6×10^{-6} M with L-glutamate and 1.4×10^{-6} M with γ-HG; for α-methylglutarate, the value was 8.4×10^{-3} M for either of the two substrates. These values were obtained using enzyme fraction VI (Table I), having a specific activity of 6550.

Comparison of K_m and V_{max} Values for Glutamate and γ-HG. The apparent Michaelis constants and relative maximum velocities were determined with either L-glutamate or *threo*-DL-γ-HG as substrate. K_m values were obtained from Lineweaver–Burk (1934) double-reciprocal plots. These experiments were carried out with fraction VI (Table I) of the decarboxylase having a specific activity equal to 7241. L-Glutamate was found to have a K_m value of 6.7×10^{-3} M compared to a value of 1.9×10^{-2} M for *threo*-DL-γ-HG. Under the same conditions, the V_{max} (defined in this instance on a uniform basis as microliters of carbon dioxide liberated per minute per milligram of protein) for L-glutamate was 1.38 and that for *threo*-DL-γ-HG was 0.05.

Stoichiometry of γ-HG Decarboxylation. The stoichiometric conversion of synthetic *threo*-DL-γ-HG by the partially purified decarboxylase to carbon dioxide and α-hydroxy-γ-aminobutyric acid was shown as follows. Fraction VI (Table I, 0.3 mg of protein) was used as the source of enzyme in these experiments. The incubation mixture contained all of the usual components present in the standard assay for γ-HG decarboxylation in a final volume of 3.0 ml. Decarboxylation was allowed to proceed for 90 min at 37°; the reaction was then stopped by the addition of 0.5 ml of concentrated HCl. Manometric measurement showed the release of 9.1 μmoles of CO_2. The γ-HG not utilized as substrate and the α-hydroxy-γ-aminobutyric acid liberated as product were then removed from the deproteinized reaction mixture by passing the solution over a column (1.5 × 25 cm) of Dowex 50 (H^+) resin. Thorough washing of the column removed anionic and neutral materials and the amino acids were subsequently eluted with 2 N NH_4OH solution. Repeated concentration of the eluate to dryness *in vacuo* removed the ammonia. The pH of the resulting solution was then adjusted to 7.0 by the addition of alkali and the two amino acids were fractionated on a column (1.5 × 25 cm) of Dowex 1 (acetate) resin. α-Hydroxy-γ-aminobutyric acid was removed by washing with water, and γ-HG was eluted with 0.5 M acetic acid. The wash fluid and eluate were concentrated to dryness under reduced pressure in separate flasks, the residues were dissolved in water, and the concentration of each amino acid was determined by quantitative reaction with ninhydrin (Rosen, 1957). Paper chromato-

TABLE III: Paper Chromatographic Properties of Homoserine, α-Hydroxy-γ-aminobutyric Acid (α-HO,GABA), and the Oxidation Product of the Enzymatically Formed Compound.[a]

			R_F Values					
	Solvent	Ratio	Ref α-HO,-GABA	Oxidation Mixture of the Enzymatic Product and of Ref α-HO,GABA	Ref β-Alanine	Ref Homoserine	Oxidation Mixture of Ref Homoserine	Ref Aspartic Acid
A	Phenol saturated with citrate–phosphate buffer[b]		0.51	0.52,0.65	0.62	0.50	0.50,0.05	0.09
B	1-Butanol–acetic acid–H_2O	4:1:1	0.53	0.53,0.79	0.79	0.55	0.51,0.38	0.36
C	Pyridine–acetic acid–H_2O	10:7:3	0.28	0.28,0.36	0.36	0.30	0.30,0.20	0.20
D	*t*-Butyl alcohol–formic acid–H_2O	14:3:3	0.38	0.40,0.46	0.46	0.40	0.40,0.32	0.32
E	Phenol–ammonia	200:1	0.54	0.54,0.61	0.60	0.54	0.54,0.15	0.17

[a] Whatman No. 1 filter paper, ascending solvent flow was used with solvents A, C, and E; Whatman No. 3 filter paper, descending fluid flow was used for solvents B and D. Compounds were detected by spraying with a 0.25% solution of ninhydrin in a 50:50 mixture of pyridine–water. [b] Phenol saturated with an aqueous solution containing 6.3% sodium citrate and 3.7% sodium dihydrogen phosphate.

graphic examination of each solution demonstrated the presence of a small amount of γ-HG in the water wash fluid; appropriate corrections were applied. The results showed the formation of 10.9 μmoles of α-hydroxy-γ-aminobutyric acid and the disappearance of an equimolar amount of γ-HG, in agreement with the generally accepted stoichiometry of decarboxylation reactions.

Identification and Properties of α-Hydroxy-γ-aminobutyric Acid. Theoretically, the decarboxylation of γ-HG can occur in one of two ways, either by loss of the α-carboxyl group with the formation of α-hydroxy-γ-aminobutyric acid, or by removal of the γ-carboxyl group and the liberation of homoserine. In this instance, the enzyme catalyzes the liberation of carbon dioxide from the α-carboxyl group. This was shown as follows.

A large incubation mixture (containing all the components in the usual assay increased by a factor of 10; fraction V (Table I) served as the source of enzyme) was incubated at 37° for 5 hr. The reaction was stopped by adding an equal volume of acetone, the precipitated protein was removed by centrifugation, and the acetone was removed from the supernatant fluid by concentrating *in vacuo*. The hydroxyamino acid formed as the product was isolated by ion-exchange chromatography on columns of Dowex 50 (H^+) and Dowex 1 (acetate) resin, as just described in the preceding section. If any traces of unreacted γ-HG were detected in the water wash from the Dowex 1 (acetate) column, these traces were removed either by a second passage of this solution over a Dowex 1 (acetate) column or by preparative paper chromatography. The isolated product, homogeneous in a wide range of paper chromatographic solvent systems, was used in the following tests.

As seen in Tables III and IV, none of the chromatographic solvent systems used resolved known samples of α-hydroxy-γ-aminobutyric acid and homoserine. More definitive results were obtained in this manner. After chromatography of the enzymatic product with the two reference compounds in any of the solvent systems listed, the paper sheet was first sprayed either with $Cu(NO_3)_2$ solution (Larsen and Kjaer, 1960) or with pyridoxal and then heated, as described by Kalyankar and Snell (1957). When the chromatogram was finally sprayed with ninhydrin solution, only reference α-hydroxy-γ-aminobutyric acid and the isolated enzymatic product uniformly reacted to give the typical violet-purple color.

Further proof was obtained by identifying the oxidation and the reduction product of the enzymatically formed amino acid. Approximately 50 μmoles (6 mg) of the isolated product and 3.75 mg of $KMnO_4$ were dissolved in 95 μl of water and 45 μl of 20% H_2SO_4. Oxidation was carried out by shaking this mixture for 2 hr at 10°. In order to reduce the enzymatic product, about 10 μmoles (1 mg) of the compound, 1 mg of dry, red phosphorus, and 10 μl of 47% HI were heated in a sealed tube for 4 hr at 140°. Reference samples of homoserine and α-hydroxy-γ-aminobutyric acid were oxidized and reduced in an identical manner. In both cases (oxidation and reduction), the reaction mixture was transferred to a column of Dowex 50 (H^+) resin, and after washing the columns well with water the amino acids were eluted

TABLE IV: Paper Chromatographic Properties of Homoserine, α-Hydroxy-γ-aminobutyric Acid (α-HO,GABA), and the Reduction Product of the Enzymatically Formed Compound.[a]

			R_F Values					
	Solvent	Ratio	Ref α-HO,-GABA	Reduction Mixture of the Enzymatic Product and of Ref α-HO,GABA	Ref γ-Amino-butyric Acid	Ref Homo-serine	Reduction Mixture of Ref Homo-serine	Ref α-Amino-butyric Acid
A	Phenol saturated with citrate–phosphate buffer[b]		0.52	0.52,0.78	0.79	0.54	0.54,0.66	0.67
B	Methanol–1-butanol–benzene–H_2O	2:1:1:1	0.30	0.29,0.36	0.36	0.32	0.33,0.46	0.46
C	Ethanol–H_2O	7:3	0.40	0.40,0.46	0.46	0.44	0.43,0.56	0.56
D	*t*-Butyl alcohol–formic acid–H_2O	14:3:3	0.38	0.38,0.50	0.50	0.42	0.42,0.61	0.60

[a] Whatman No. 1 filter paper, ascending solvent flow was used with solvents A–C; Whatman No. 3 filter paper, descending fluid flow was used for solvent D. Compounds were detected by spraying with a 0.25% solution of ninhydrin in pyridine–water (50:50). [b] Phenol saturated with an aqueous solution containing 6.3% sodium citrate and 3.7% sodium dihydrogen phosphate.

with 2 N NH_4OH solution. Repeated concentration *in vacuo* of the eluate removed the ammonia and yielded a concentrated solution which was examined by paper chromatographic methods. Tables III and IV show that the enzymatic product is oxidized to β-alanine and yields γ-aminobutyric acid when reduced.

Finally, when [5-^{14}C]γ-HG was decarboxylated by the enzyme, unlabeled carbon dioxide was released and a radioactive product was formed that migrated identically with reference α-hydroxy-γ-aminobutyric acid on two-dimensional chromatograms (Figure 2) and also in a variety of one-dimensional paper chromatographic solvent systems (see Tables III and IV). Collectively, these results show that the product formed by action of the bacterial decarboxylase on γ-HG is α-hydroxy-γ-aminobutyric acid.

Specificity of the Decarboxylase for Isomeric Forms of γ-HG. As noted frequently before, *threo*-DL-γ-HG was used almost exclusively in most of the studies reported. When tested with the individual isomers of γ-HG, the purified decarboxylase showed a high degree of specificity for only the *threo*-L form (Figure 3). Approximately the same initial rate of reaction is observed with the *threo*-L isomer as with twice the molar concentration of the *threo* racemate. In contrast, the *erythro* racemate or the *erythro*-L isomer are not utilized as substrates to any significant extent.

Specificity of the Decarboxylase for Glutamic Acid Analogs and Other Compounds. A wide variety of analogs of glutamic acid were tested as possible substrates for the decarboxylase. Of 27 compounds tested, only 8 showed a significant level of carbon dioxide formation when incubated with the decarboxylase under routine assay conditions. The activity of these compounds a compared to L-glutamate is shown in Table V. Assum ing that L-glutamine is not a direct substrate but is either contaminated by small amounts of glutamic acid or is slowly hydrolyzed to the free amino acid, *threo*-DL-γ-HG ranks as the third best substrate after L-glutamate.

TABLE V: Analogs of Glutamic Acid as Substrates for the Decarboxylase.[a]

	CO_2 Liberated (μl/60 min per mg of protein)
L-Glutamate	16,200[b]
γ-Methylene-DL-glutamate	4,775[b]
threo-β-Hydroxy-DL-glutamate	2,865[b]
erythro-β-Hydroxy-DL-glutamate	100
L-Glutamine	333
threo-γ-Hydroxy-DL-glutamate	330
erythro-γ-Hydroxy-DL-glutamate	0
γ-Benzyl-L-glutamate	200
γ-Methyl-DL-glutamate	60
γ-L-Glutamyl methyl ester	60

[a] Enzyme corresponding to fraction V (Table I) was used. The compounds were tested at these levels (μmoles/3 ml): L-glutamate, 25; L-glutamine and γ-L-glutamyl methyl ester, 50; all others, 100. [b] Values obtained by extrapolation of shorter reaction times.

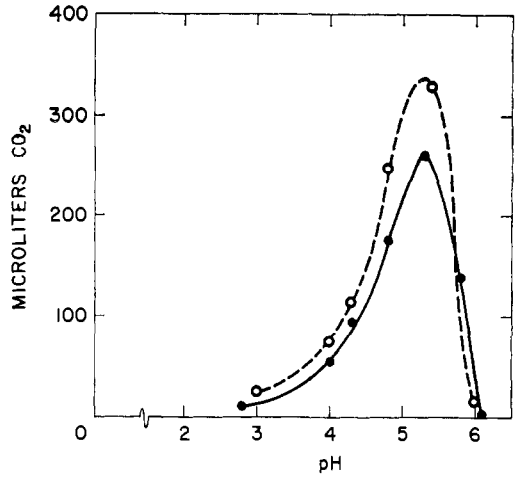

FIGURE 1: Optimum pH for decarboxylation of either L-glutamate or *threo*-DL-γ-HG. The reaction mixtures contained the components listed in the section, Enzyme Assay. Only citric acid–sodium phosphate buffers were used. Fraction VI (Table I) served as the source of the enzyme; with L-glutamate as substrate, 0.029 mg of protein was added; for *threo*-DL-γ-HG, 0.29 mg of protein. (O– – –O) γ-Hydroxyglutamate and (●—●) L-glutamate.

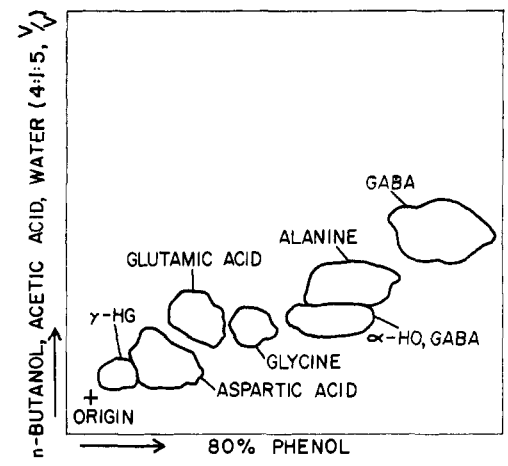

FIGURE 2: Tracing of two-dimensional (23 × 23 cm) chromatogram and radioautogram of glutamic acid, γ-HG, and related catabolic products. Order of solvents: 80% phenol followed by 1-butanol–acetic acid–water. Ninhydrin-positive areas were visualized by spraying with 0.25% ninhydrin solution in pyridine–H_2O (50:50); radioactive spots (γ-HG and α-HO, GABA) were detected by exposure to X-ray film for 2 weeks. γ-HG = γ-hydroxyglutamic acid; α-HO-GABA = α-hydroxy-γ-aminobutyric acid; GABA = γ-aminobutyric acid.

γ-Methylene-DL-glutamate and *threo*-β-hydroxy-DL-glutamate are considerably better substrates for decarboxylation than is *threo*-DL-γ-HG.

The following compounds showed no release of carbon dioxide when tested manometrically with the bacterial decarboxylase: *N*-acetyl-L-glutamate, *p*-aminobenzoyl-L-glutamate, α,γ-diaminoglutarate, carbamyl-L-glutamate, γ-L-glutamyl ethyl ester, γ-L-glutamylhydrazide, α-L-glutamyl methyl ester, D-glutamate, D-glutamine, α-hydroxy-L-glutarate, L-pyrrolidonecarboxylic acid, L-aspartate (all tested individually at a final concentration of 50 μmoles/3 ml), and γ-ethyl-DL-glutamate, γ-hydroxy-γ-methyl-DL-glutamate, β,γ-dihydroxyglutamate, α-methyl-DL-glutamate, β-methyl-DL-glutamate, *erythro*-β-hydroxy-DL-aspartate, and *threo*-β-hydroxy-DL-aspartate (each tested at a concentration of 100 μmoles/3 ml).

Discussion

The various independent lines of evidence presented in this paper support the conclusion that γ-HG is decarboxylated by glutamate decarboxylase of *E. coli*. By inference, one would assume that the γ-hydroxyamino acid also serves as substrate for brain glutamate decarboxylase. This conclusion contrasts with reports documenting the presence in bacteria (Umbreit and Heneage, 1953) and in brain (Hisada and Nakashima, 1960) of a specific decarboxylase for β-hydroxyglutamate, different from glutamate decarboxylase. It is interesting to note, however, that our preparation of partially purified decarboxylase from *E. coli* (ATCC 11246) exhibits a very significant degree of activity toward the β-hydroxyamino acid (Table V); preferential use of the *threo* isomer as substrate agrees with the results of Umbreit and Heneage (1953).

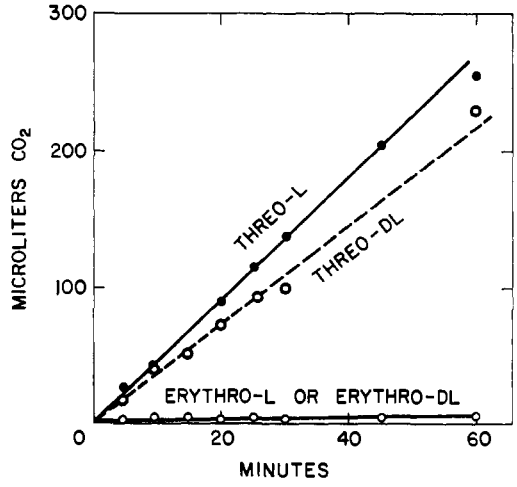

FIGURE 3: Optical isomers of γ-HG as substrates for the decarboxylase. The reaction mixtures contained the usual components described in the Enzyme Assay section; 50 μmoles of the respective L isomer and 100 μmoles of the racemic mixture were added. Fraction V (Table I, 0.69 mg of protein) served as the source of the enzyme.

Since mammalian liver contains very little or no glutamate decarboxylase activity and, by inference from the results presented in this paper, it is glutamate decarboxylase in mammalian tissues (brain) that utilizes γ-HG as substrate, one would not expect to observe a decarboxylation of the hydroxyamino acid by liver preparations. We confirmed this expectation; we could not detect any decarboxylase activity toward γ-HG in a number of different kinds of liver extracts. The absence of γ-HG decarboxylase activity in rat liver was previously reported by Bouthillier and Binette (1961). Kuratomi *et al.* (1963), however, indicated that rat liver homogenates catalyze a very significant rate of γ-HG decarboxylation. Since no supporting data, providing details on the nature of the liver homogenates used and the conditions for measuring the enzymatic reaction, were provided by the latter investigators, we cannot at this time explain the discrepancy between their findings and our negative results in this regard. As it is well established that 2-keto-4-hydroxyglutarate, glyoxylate (thence glycine), and pyruvate (thence alanine) are primary metabolic products derived from γ-HG in liver, we also examined these compounds as possible substrates for decarboxylation; we detected no such activity in rat liver preparations. The possibility that γ-HG is metabolically converted by liver to some other product which is subsequently decarboxylated is, therefore, also eliminated.

γ-HG has a lower apparent affinity for the decarboxylase than does L-glutamate. This difference is not immediately apparent from the individual specific activity values listed for the two substrates nor the ratios calculated in Table II, but it should be recalled that ten times as much protein and a much longer reaction time (60 *vs.* 10 min) was used with γ-HG as substrate.

The specificity of glutamate decarboxylase from extracts of *E. coli* (ATCC 11246) for the *threo*-L isomer of γ-HG is unique among those enzymes currently known to utilize γ-HG as substrate. Highly purified glutamate–aspartate transaminase from either rat liver or pig heart extracts catalyzes the transamination of either the *erythro*-L or the *threo*-L isomers; the former isomer, however, is the more effective (Maitra and Dekker, 1964). Likewise, both L isomers of γ-HG are amidated almost equally well by highly purified rat liver glutamine synthetase, with possibly a slight preference for the *erythro* isomer (Goldstone and Adams, 1965). In mammals, *erythro*-L-γ-HG is formed from hydroxy-L-proline (Adams and Goldstone, 1960a) whereas the *threo*-L isomer is native to the plant *Phlox decussata* (Dekker, 1962). Mouse brain β-hydroxyglutamate decarboxylase is only active with the *erythro*-L isomer (Nakashima, 1961); the same enzyme in extracts of a number of strains of *E. coli* preferentially utilizes *threo*-β-hydroxy-L-glutamate (Umbreit and Heneage, 1953). On the other hand, *erythro*-5-hydroxy-L-lysine is a better substrate for lysine decarboxylase of *Bacterium cadaveris* than is the *threo*-L form (Linstedt, 1951; Linstedt and Linstedt, 1962).

The decarboxylation of γ-HG by bacterial and brain preparations raises the question as to what the role of the resulting product, α-hydroxy-γ-aminobutyrate, may be. A particularly interesting point awaiting further investigation is the question of what pharmacological properties this compound may have in the central nervous system of mammals. The very marked effects exhibited by γ-aminobutyrate and by β-hydroxy-γ-aminobutyrate in this regard and the possible role compounds of this nature play in nerve tissues have recently been reviewed in detail (Curtis and Watkins, 1965). α-Hydroxy-γ-aminobutyric acid is also the initial product formed by a soil bacterium, *Agrobacterium* sp., which utilizes azetidine-2-carboxylic acid as the sole source of nitrogen (Dunnill and Fowden, 1965).

The data reported here regarding the specificity of glutamate decarboxylase for glutamic acid analogs and related compounds must be regarded as only a beginning. Desirably, ultimate efforts along this line await the use of homogeneous decarboxylase preparations. Certain tentative and interesting points for glutamate analogs, however, may already be noted. (a) A free, unblocked α-amino group is required. α-Hydroxyglutarate, *N*-acetyl-L-glutamate, carbamyl-L-glutamate, *p*-aminobenzoyl-L-glutamate, and L-pyrrolidonecarboxylic acid are not decarboxylated. (b) Substitution by certain groups in the γ-position does not completely block decarboxylase activity. γ-Methylene-, γ-hydroxy-, γ-benzyl-, and γ-methylglutamates are utilized as substrates to a greater or lesser extent. γ-Aminoglutamate (α,γ-diaminoglutarate), γ-hydroxy-γ-methylglutamate, and γ-ethylglutamate, in contrast, are not. Just recently, Fowden (1966) also reported that γ-ethylideneglutamate is not decarboxylated. (c) Methyl group substitution in the α and β positions is more deleterious than is substitution of the same group in the γ position. (d) Blocking of either carboxyl group drastically reduces or completely blocks decarboxylase activity. Further studies along this line with pure enzyme and specific isomers of each glutamate analog should provide interesting information concerning the binding site(s) and conformation of the active site of the decarboxylase.

Acknowledgement

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CORRECTION

In the paper "The Intracellular Distribution of Polynucleotide Phosphorylase in *Escherichia coli* Cells," by Y. Kimhi and U. Z. Littauer, on page 2067, line 13 from the bottom, left-hand column, $MgCl_2$, 0.05; EDTA (pH 8.0), 0.005, should be changed to $MgCl_2$, 0.5; EDTA (pH 8.0), 0.05.