Isolation and Properties of γ-L-Glutamylcyclotransferase from Human Brain*

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ABSTRACT: γ-L-Glutamylcyclotransferase, which catalyzes the conversion of certain γ-L-glutamyl-L-amino acids into pyrrolidonecarboxylic acid and amino acid, has been purified more than 1000-fold from human and sheep brain.

A convenient assay has been devised in which γ-L-glutamyl-γ-L-glutamyl-*p*-nitroanilide is used as the substrate. The enzyme converts this substrate into pyrrolidonecarboxylic acid and γ-L-glutamyl-*p*-nitroanilide; the latter compound is much more labile to alkali than the substrate and therefore, after standardized alkaline hydrolysis, the product may be quantitatively determined. The enzymatic reaction has also been followed by a new assay for pyrrolidonecarboxylic acid based on its high absorbance at 205 mμ. The purified enzyme acts on the γ-L-glutamyl derivatives of L-glutamine, L-alanine, L-α-aminobutyric acid, and glycine, but is inactive toward glutathione, γ-glutamyl-*p*-nitroanilide, and certain other γ-glutamylamino acids. Studies carried out with mixtures of γ-glutamylcyclotransferase and γ-glutamyltranspeptidase suggest that the previously observed conversion of glutathione into pyrrolidonecarboxylate involves the intermediate formation of γ-glutamylglutathione. Several possible metabolic functions of the enzyme are considered.

γ-Glutamylcyclotransferase, which occurs in a number of animal tissues, catalyzes the conversion of γ-glutamylamino acids into pyrrolidonecarboxylic acid and free amino acid in accordance with the following reaction

γ-L-glutamyl-L-amino acid → L-pyrrolidonecarboxylic acid + L-amino acid

This enzyme activity was first observed by Connell and Hanes (1956), who obtained a partially purified preparation of it from pig liver; this preparation acted on γ-glutamylglycine, γ-glutamylglutamic acid, and, more slowly, on γ-glutamylphenylalanine and glutathione.

In the present work an attempt has been made to obtain a highly purified preparation of the enzyme. It was found that a protein fraction obtained in the course of purification of glutamine synthetase from sheep brain (Pamiljans *et al.*, 1962), and normally discarded during the isolation of this enzyme, was particularly rich in γ-L-glutamylcyclotransferase activity. We have therefore prepared and used this fraction for the purification of the γ-glutamylcyclotransferase of sheep and human brain; preparations that are more than 1000-fold purified have been obtained. In the course of this work a convenient method of assay has been developed in which γ-L-glutamyl-γ-L-glutamyl-*p*-nitroanilide is used as the substrate. The enzyme converts this compound into pyrrolidonecarboxylic acid and γ-L-glutamyl-*p*-nitroanilide. γ-L-Glutamyl-*p*-nitroanilide is much more labile to alkali than is γ-L-glutamyl-γ-L-glutamyl-*p*-nitroanilide, and it is therefore possible to obtain the per cent conversion of the substrate into its products by determining *p*-nitroaniline after alkaline hydrolysis under standardized conditions. The reaction has also been followed by means of a new quantitative assay method for pyrrolidonecarboxylic acid based on the high absorbance of this compound below 220 mμ. Purified preparations of γ-glutamylcyclotransferase from brain act on γ-glutamylglutamine, γ-glutamylalanine, γ-glutamylglycine, and several other γ-glutamylamino acids. The enzyme is inactive toward certain γ-glutamylamino acids and does not act to an appreciable extent on glutathione or γ-glutamyl-*p*-nitroanilide; however, in the presence of γ-glutamyltranspeptidase, glutathione, γ-glutamyl-*p*-nitroanilide, and a number of γ-glutamylamino acids which are very poor substrates for the γ-glutamylcyclotransferase are rapidly converted into pyrrolidonecarboxylic acid. The findings suggest that γ-glutamylglutathione and other γ-glutamyl-γ-glutamylamino acids are substrates for γ-glutamylcyclotransferase.

Experimental Section

Materials

γ-L-Glutamyl-γ-L-glutamyl-*p*-nitroanilide was synthesized by treating γ-L-glutamyl-*p*-nitroanilide (Orlowski and Meister, 1965) with phthaloyl-L-glutamic acid anhydride (Sheehan and Bolhofer, 1950; King *et al.*, 1957) by a modification of the general method of King and Kidd (1949). Phthaloyl-L-glutamic anhydride (7.8 g, 0.03 mole) was dissolved in 30 ml of hot glacial acetic acid. γ-L-Glutamyl-*p*-nitroanilide (8.4 g, 0.0295 mole) was then added and the mixture was heated rapidly to 100°. After 0.5 min the mixture was allowed to cool to about 26°. The acetic acid was removed by evaporation under reduced pressure and the residue

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thus obtained was dissolved in methanol. The methanol was then removed by evaporation under reduced pressure and the residue was redissolved in methanol. This process was repeated several times until a semisolid product remained. This was dissolved in 80 ml of methanol containing 6.07 g of triethylamine; 20 ml of methanol containing 2.8 g of 80% hydrazine was then added. After standing for 2 days at 26°, 2 ml of glacial acetic acid was added and the mixture was allowed to stand at 4° for 2 days. The precipitate which formed was removed by filtration, and the filtrate was then evaporated to dryness under reduced pressure. The residue was suspended in the minimal amount of water and adjusted to about pH 1 by addition of 75 ml of 2 N HCl. This solution was filtered and the filtrate was shaken with one-half volume of ethyl acetate. The aqueous layer was separated and adjusted to pH 3 by addition of a saturated solution of sodium acetate. This solution was placed at 4°; the product, which crystallized over a period of several days, was recrystallized from 10% acetic acid. Further purification was effected by dissolving the product (4 g) in 20 ml of 0.5 M Tris and adding a mixture of ethanol (30 ml) and glacial acetic acid (20 ml). After standing overnight at 4°, the crystalline product was collected on a Büchner funnel and washed with ethanol. The crystals were dried *in vacuo* over calcium chloride and sodium hydroxide. The yield was 2.6 g (20%): mp 165–168°, $[\alpha]_D^{20}$ +10.5°. *Anal.* Calcd for $C_{16}H_{20}N_4O_8 \cdot 2H_2O$: C, 44.4; H, 5.6; H_2O, 8.33; N, 13.0. Found: C, 44.5; H, 5.5; H_2O, 8.60; N, 12.8.

The other three stereoisomers of γ-glutamyl-γ-glutamyl-*p*-nitroanilide, *i.e.*, the LD, DD, and DL forms, were prepared by the procedure described above by appropriate use of phthaloyl-L- or D-glutamic anhydride and D- or L-γ-glutamyl-*p*-nitroanilide. These compounds gave single ninhydrin-positive spots on descending chromatography on Whatman No. 1 paper in the following solvents: 1-butanol–pyridine–water (1:1:1, v/v) and 1-butanol–acetic acid–water (60:15:25, v/v). The R_F values were, respectively, 0.53 and 0.57. All of the γ-glutamyl-γ-glutamyl-*p*-nitroanilide stereoisomers exhibited maximum absorbance at 315 mμ and a molar extinction coefficient of 13,200; they did not absorb at 410 mμ, at which wavelength the absorbance of free *p*-nitroaniline can be measured.

γ-L-Glutamylglycine, γ-L-glutamylglycine ethyl ester, γ-L-glutamyl-β-alanine, and γ-L-glutamyl-L-leucine were prepared by the method of LeQuesne and Young (1950). γ-L-Glutamyl-L-alanine, γ-L-glutamyl-L-valine, γ-L-glutamyl-L-phenylalanine, γ-L-glutamyl-L-tyrosine, γ-L-glutamyl-*S*-methyl-L-cysteine, and γ-L-glutamyl-β-aminoisobutyric acid were generously provided by Dr. John F. Thompson. L-Glutamine, reduced glutathione, and oxidized glutathione were obtained from Mann Research Laboratories.

γ-L-Glutamyl-L-glutamine was prepared by the action of hog kidney γ-glutamyl transpeptidase (Orlowski and Meister, 1965) on glutathione in the presence of L-glutamine as follows. Glutathione (3.073 g, 10 mmoles) and L-glutamine (4.386 g, 30 mmoles) were dissolved in water and adjusted to pH 8.7 by addition of sodium hydroxide; the final volume was brought to 200 ml, and 0.5 ml of purified γ-glutamyl transpeptidase (50 units) was added. The solution was incubated at 37° for 1 hr after which it was placed at 0°. The product was isolated by chromatography on a column of Dowex 1 acetate (X8, 200–400 mesh, 4 × 75 cm) prepared as described by Cohn (1957). The cooled reaction mixture was added at 5° to the top of the column, which was then washed with 8 l. of water. Elution was carried out in sequence with 4 l. each of acetic acid solutions of the following concentrations: 0.05, 0.10, 0.15, and 0.30 M (Morris *et al.*, 1964). γ-Glutamylglutamine was eluted with 0.30 M acetic acid. The fractions containing the product were combined and evaporated *in vacuo* (below 40°) to low volume. This solution was lyophilized and the product was then crystallized from water–ethanol. The yield was 1.1 g, mp 194–195° dec; 193–194° (Kakimoto *et al.*, 1967). On paper electrophoresis at pH 5.0 (0.05 M sodium acetate buffer) at 62.5 V/cm, the product moved as a single spot 13.8 cm toward the anode; the comparable mobilities of glutamine and glutamic acid, were, respectively, 0.85 and 16.9 cm. On prolonged incubation with γ-glutamylcyclotransferase, the product was completely converted into glutamine and pyrrolidonecarboxylate. After acid hydrolysis (4 N HCl, 100°, 4 hr) glutamic acid was found as the only amino acid product. On descending paper chromatography with a solvent consisting of butanol–pyridine–water (1:1:1, v/v), the product exhibited a R_F value of 0.17 compared with values of 0.21 and 0.26 for glutamic acid and glutamine, respectively.

N-Carbobenzoxy-α,γ-L-glutamyldiglycine ethyl ester was prepared as follows. *N*-Carbobenzoxy-L-glutamic acid (5.65 g, 20 mmoles) was dissolved in tetrahydrofuran (100 ml); glycine ethyl ester hydrochloride (5.6 g) was added followed by triethylamine (4.05 g). The suspension was stirred in an ice bath for several minutes. Dicyclohexylcarbodiimide (8.66 g) was added and the mixture was stirred at 26° for 2 days. Then 0.3 ml of glacial acetic acid was added and the precipitate was removed by filtration and washed with tetrahydrofuran. The combined filtrate and washings were evaporated under reduced pressure to yield a white solid which was dissolved in 50 ml of chloroform. The chloroform solution was washed successively with 50 ml of each of the following solutions: 1 N HCl, water, 1 M $KHCO_3$, and water. The chloroform solution was dried over anhydrous sodium sulfate and then evaporated to dryness under reduced pressure. The residue was dissolved in a small volume of chloroform; on addition of petroleum ether (bp 30–60°) a white crystalline product was obtained: yield, 4.8 g (53%). *Anal.* Calcd for $C_{21}H_{28}N_3O_8$: C, 55.9; H, 6.5; N, 9.3; Found: C, 57.0; H, 6.9; N, 9.43.

α,γ-L-Glutamyldiglycine was prepared from *N*-carbobenzoxy-α,γ-L-glutamyldiglycine ethyl ester as follows. *N*-Carbobenzoxy-α,γ-L-glutamyldiglycine ethyl ester (1.35 g, 3 mmoles) was dissolved in 27 ml of dioxane; 6 ml of water and 3 ml of 2 N sodium hydroxide were then added. The solution was allowed to stand at 26° for 1 hr, after which 0.3 ml of 2 N sodium

TABLE I: Summary of Purification of γ-Glutamylcyclotransferase from Human Brain.

Step	Vol. (ml)	Total Protein (mg)	Protein Concn (mg/ml)	Total Act. (units)	Sp Act. (units/mg)	Yield (%)
1. Acetone powder extract	1,464	20,496	14.0	2,020	0.098	[100]
2. Precipitation at pH 4.2	1,500	10,275	6.85	1,950	0.190	97
3. Ammonium sulfate fractionation	76	4,408	58.0	1,940	0.440	96
4. Selective heat denaturation	55	2,172	39.5	1,727	0.790	85
5. Gel filtrations, Sephadex G-75	243	182	0.75	1,397	7.67	69
6. Carboxymethyl cellulose column	248	96.7	0.39	1,300	13.4	64
7. Gel filtration, Sephadex G-75	41	30.4	0.76	1,035	34.0	51
8. DEAE-cellulose chromatography	110	5.5	0.05	550	100	27

hydroxide was added and the mixture was allowed to stand for an additional 2 hr at 26°. It was then brought to pH 7 by addition of an equivalent amount of 2 N HCl. The solution was evaporated to dryness; the residue was suspended in 20 ml of water and then heated at 80° for 5 min. The solution was filtered and the filtrate was evaporated under reduced pressure to yield a residue which was dissolved in ethanol. This solution was filtered to remove sodium chloride and the filtrate was evaporated under reduced pressure to yield an oil which solidified on addition of ethyl acetate. The yield was approximately 0.7 g. Catalytic hydrogenation was then carried out with palladium catalyst in methanol. The solvent was removed under reduced pressure, and the residue was suspended in ethyl acetate and petroleum ether. The solvents were removed under reduced pressure to give a solid residue, which was crystallized from methanol. *Anal.* Calcd for $C_9H_{15}N_3O_6$: C, 41.4; H, 5.8; N, 16.1. Found: C, 40.7; H, 5.8; N, 15.2.

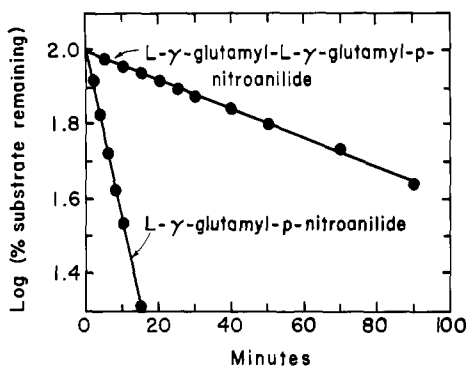

FIGURE 1: Release of *p*-nitroaniline from γ-L-glutamyl-γ-L-glutamyl-*p*-nitroanilide and γ-L-glutamyl-*p*-nitroanilide in 0.1 N NaOH at 37°; the concentration of γ-glutamyl compounds was 0.2 mM.

Methods

Determination of Enzyme Activity. Two methods were used for the determination of enzymatic activity. The first of these is based on the fact that γ-L-glutamyl-*p*-nitroanilide hydrolyzes considerably more rapidly in alkali than does γ-L-glutamyl-γ-L-glutamyl-*p*-nitroanilide; thus, the time intervals required for 50% hydrolysis in 0.1 N NaOH at 37° are 6.4 and 76.6 min, respectively (Figure 1). γ-L-Glutamyl-γ-L-glutamyl-*p*-nitroanilide (172.8 mg) was suspended in 0.8 ml of 0.5 M sodium bicarbonate and dissolved by bringing the final volume to 10 ml with water. The enzyme solution (0.1 ml) was added to a mixture of 0.1 ml of substrate solution (containing 4 μmoles) and 0.3 ml of Tris-HCl buffer (20 μmoles, pH 8.0). After incubation at 37° for 0, 10, and 20 min, aliquots (0.05 ml) of the reaction mixture were removed and added at 37° to small test tubes containing 1.75 ml of 0.1029 M sodium hydroxide. After exactly 20 min, 0.2 ml of 10 M acetic acid was added to each test tube and the absorbance of free *p*-nitroaniline was determined at 410 mμ. After subtraction of the zero time absorbance value, the corrected absorbance value was used to calculate the concentration of γ-glutamyl-*p*-nitroanilide; the millimolar concentration of γ-glutamyl-*p*-nitroanilide is equal to the absorbance at 410 mμ divided by 6.47. The formation of γ-L-glutamyl-*p*-nitroanilide was linear with time until about 50% of the substrate was used. A unit of enzyme is defined here as that amount which catalyzes the formation of 1 μmole of γ-glutamyl-*p*-nitroanilide/min under the conditions given above. Specific activity is expressed in terms of units per milligram of protein, determined as described by Lowry *et al.* (1951).

Enzymatic activity may also be determined by measuring the formation of pyrrolidonecarboxylic acid. In the method described here advantage is taken of the high absorbance exhibited by pyrrolidonecarboxylic acid below 220 mμ, due to its peptide bond. This method is applicable in principle to any of the substrates of the enzyme; however, interfering substances must be removed. This was accomplished by passing the reaction mixtures through columns of Dowex 50 (H^+). Pyrrolidonecarboxylic acid is not absorbed by the column and its concentration may be determined in the eluate from the absorbance at 205 mμ. Under the conditions described here as little as 0.02 μmole of pyrrolidonecarboxylic acid may be accurately determined. Dowex 50-X4 (200–400 mesh) was used; the resin was washed in sequence with acetone, petroleum

ether (bp 40–60°), acetone, 2 N HCl, 2 N NaOH, 2 N HCl, and water. The final washing with water was continued until no further ultraviolet-absorbing material was released. Small columns (1 × 2.5 cm) equipped with sintered glass bottoms were filled with about 1 ml of packed resin. A separate column was used for each determination. The enzyme solution (0.05 ml) was added to 0.2 ml of a solution containing substrate (4 μmoles) and Tris-HCl buffer (10 μmoles, pH 8.0). After incubation at 37° for 0–20 min the reaction mixture was placed at 90° for 3 min and then the incubation mixture was passed through a column. The column was washed with water until a total volume of 2.5 ml of effluent was collected. The absorbance at 205 mμ was read against a reference solution containing the same components treated in the same manner except that the enzyme was added immediately prior to heating at 90°. The formation of pyrrolidonecarboxylic acid was calculated from a standard curve prepared by carrying known amounts of authentic pyrrolidonecarboxylic acid through the assay procedure. The extinction coefficient at 205 mμ of pyrrolidonecarboxylic acid under these conditions is 2600.

Purification of the Enzyme from Human Brain. An acetone powder of human brain obtained at autopsy was prepared by the method previously described for the preparation of sheep brain acetone powder (Pamiljans *et al.*, 1962). Unless otherwise stated, the temperature was maintained at 4°.

STEP 1. ACETONE POWDER EXTRACT. Brain acetone powder (230 g) was suspended in 2 l. of a solution consisting of 0.005 M 2-mercaptoethanol and 0.15 M potassium chloride. The mixture was stirred mechanically at 26° for 30 min and was then centrifuged at 4° at 13,000*g* for 15 min. The supernatant solution was filtered through a thin layer of cotton. The filtrate was processed immediately as described below.

STEP 2. PRECIPITATION OF PROTEIN AT pH 4.2. The solution was cooled to 0° and then adjusted to pH 4.2 by adding (with mechanical stirring) 1 M acetic acid. The precipitate which formed (which contains glutamine synthetase) was removed by centrifugation at 13,000*g* for 15 min. The supernatant solution, which contains virtually all of the γ-glutamylcyclotransferase activity, was adjusted to pH 7.8 by addition of 2 N ammonium hydroxide.

STEP 3. AMMONIUM SULFATE FRACTIONATION. The solution obtained in step 2 was brought to 50% of ammonium sulfate saturation by adding solid ammonium sulfate. The resulting suspension was centrifuged at 13,000*g* for 15 min and the precipitated protein was discarded. Additional solid ammonium sulfate was added to achieve 80% of saturation and the precipitate which formed was collected by centrifugation and dissolved in about 45 ml of 0.1 M Tris-HCl buffer (pH 8.0). (It was found necessary to carry out the next step within 18 hr.)

STEP 4. SELECTIVE HEAT DENATURATION OF IMPURITIES. The solution obtained in the preceding step was placed in a flask immersed in a water bath at 80°; the enzyme solution was stirred continuously until the temperature reached 57° (about 5 min). This temperature was maintained for 5 min and the solution was then rapidly cooled in an ice bath. After centrifugation at 20,000*g* for 20 min, the precipitate was removed and the supernatant solution was processed as described below.

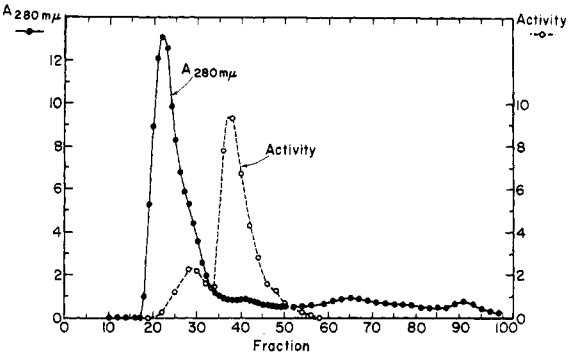

FIGURE 2: Chromatography of the enzyme on Sephadex G-75 (step 5 of the purification procedure; see the text).

STEP 5. CHROMATOGRAPHY ON SEPHADEX G-75. The solution obtained in step 4 was added to the top of a column of Sephadex G-75 (5 × 100 cm) which had been equilibrated with 0.005 M potassium phosphate buffer (pH 6.2). Elution was carried out with the same buffer and fractions of about 15 ml were collected. The major peak of activity emerged from the column shortly after appearance of a large peak containing hemoglobin and other inactive high molecular weight proteins. The activity emerged in two peaks (Figure 2), the largest of which was worked up as described below.

STEP 6. CHROMATOGRAPHY ON CM-CELLULOSE. The pooled fractions obtained in the preceding step were passed through a column of CM-cellulose (1.9 × 17 cm) which had been equilibrated with 0.005 M potassium phosphate buffer (pH 6.2). The column was eluted with the same buffer. The enzyme did not bind to the column, but a considerable amount of inactive protein remained attached to it. The enzyme solution was lyophilized and the lyophilized powder was stored at −20°.

STEP 7. CHROMATOGRAPHY ON SEPHADEX G-75. The powder obtained in step 6 was dissolved in 4 ml of 0.01 M Tris-citrate buffer (pH 8.8) and this solution was added to the top of a column of Sephadex G-75 (2 × 70 cm) which had been equilibrated with the same buffer. Elution was carried out with this buffer and fractions of about 4 ml were collected. The enzyme emerged from this column as a single peak (Figure 3).

STEP 8. CHROMATOGRAPHY ON DEAE-CELLULOSE. The enzyme solution obtained in the preceding step was passed through a DEAE-cellulose column (Whatman DE-52 microgranular, preswollen 1.9 × 12 cm) which had been equilibrated with 0.01 M Tris-citrate buffer (pH 8.8). The column was washed with 200 ml of this buffer and elution was then begun using a linear gradient established between 500 ml of 0.01 M Tris-citrate buffer (pH 8.8) and 500 ml of 0.05 M Tris-citrate buffer (pH 8.8) containing 0.15 M sodium chloride. Fractions of 10 ml were collected. The enzyme emerged

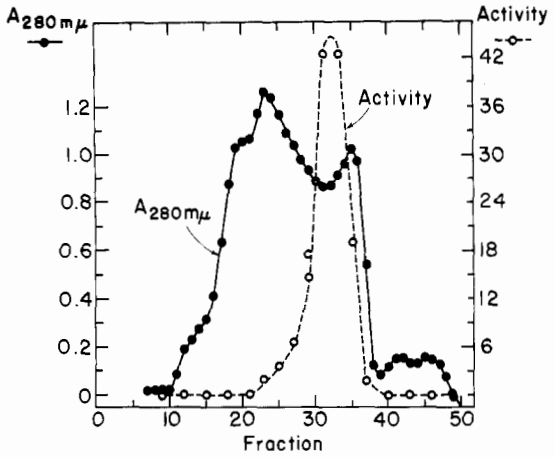

FIGURE 3: Chromatography of the enzyme on Sephadex G-75 (step 7 of the purification procedure; see the text).

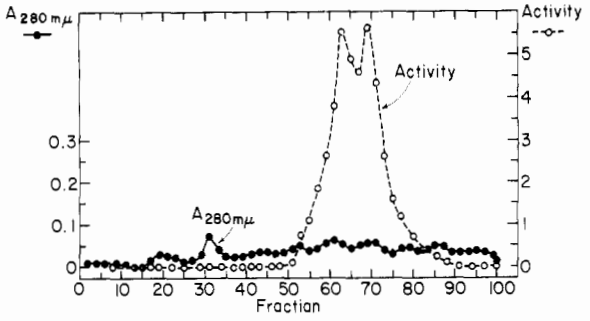

FIGURE 4: Chromatography of the enzyme on DEAE-cellulose (step 8 of the purification procedure; see the text).

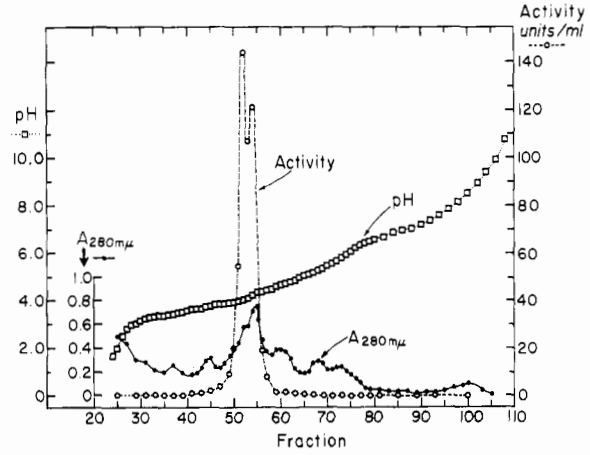

FIGURE 5: Electrofocusing of the human brain enzyme in a pH gradient (see the text).

from the column as two overlapping peaks of activity (Figure 4).

A summary of the purification is given in Table I.

The enzyme was further purified by the electrofocusing procedure (Svensson, 1961, 1962a,b; Vesterberg and Svensson, 1966) using an LKB apparatus. A pH gradient from 3.0 to 6.0, obtained with a 1% ampholine solution (LKB 8142), was established in a column (volume 400 ml) stabilized with a sucrose (5–45%) gradient. This procedure was carried out for 4 days at 4°; at equilibrium, the applied potential was 600 V and the current was 5 mA. After electrofocusing was completed, fractions of 3 ml were collected and examined for absorbance at 280 mμ, pH, and enzyme activity. The results, which are given in Figure 5, indicate the presence of two active protein fractions exhibiting isoelectric points at 4.06 and 4.25. The specific activities of these fractions were in the range 200–250, and were thus about twice that of the enzyme obtained at step 8 of the purification procedure.

Isolation of the Enzyme from Sheep Brain. Application of the procedure described above to an acetone powder of sheep brain gave a similar increase in specific activity; however, both the initial and final specific activities were about 20% of the corresponding values with human brain. The values for the acetone powder extract and the enzyme obtained on DEAE-cellulose chromatography were, respectively, 0.020 and 19.8 units per mg. Electrofocusing of the purified sheep brain enzyme was carried out in a pH gradient (5.0- to 8.0, ampholine LKB 8133, batch 4) as described above; the results are described in Figure 6. Active fractions were obtained that exhibited isolectric points at 6.20, 5.75, 5.45, 5.25, and 4.65. Several inactive protein fractions were also obtained. The most active fraction (pI 5.75) had a specific activity of 100.

Properties of the Enzyme. The enzyme activity (after step 8 and electrofocusing) was stable at 0° for a period of at least 1 month; the enzyme was also stable after lyophilization or on storage in the frozen state at −20°. Although the enzyme was stable for 5 min at 57° at step 4 of the purification procedure, the purified enzyme (after step 8) lost all activity under these conditions. The pH optimum was in the range 7.8–8.2 for both the human and sheep brain enzymes (Figure 7), and the apparent K_m value for γ-L-glutamyl-γ-L-glutamyl-*p*-nitroanilide was 4×10^{-4} M. The human brain enzyme was homogeneous in the analytical ultracentrifuge and exhibited a sedimentation coefficient, $s_{20,w}$, of 1.2 S.[1]

Substrate Specificity of the Enzyme. The activity of the purified enzymes was tested toward a number of γ-glutamyl compounds (Table II); the human brain and sheep brain enzymes exhibited similar specificity. The model substrate, γ-L-glutamyl-γ-L-glutamyl-*p*-nitroanilide, was the most active substrate; the corresponding LD, DL, and DD isomers were inactive. Substantial activity was also observed with γ-L-glutamyl-L-glutamine, γ-L-glutamyl-L-α-alanine, γ-L-glutamyl-L-α-aminobutyric acid, γ-L-glutamylglycine, and γ-L-glutamyl-*S*-methyl-L-cysteine. The other compounds examined were not active or only slightly active. However,

[1] We are indebted to Dr. Rudy H. Haschemeyer for the ultracentrifugal studies.

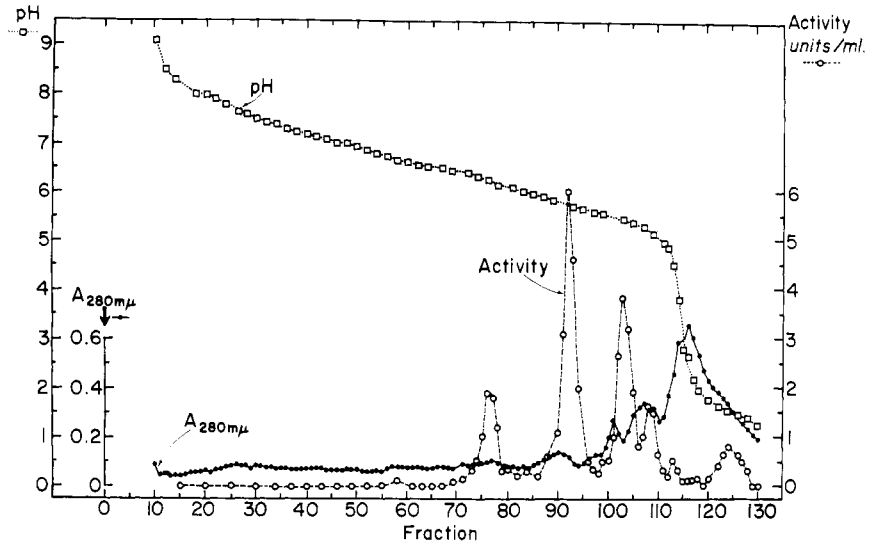

FIGURE 6: Electrofocusing of the sheep brain enzyme in a pH gradient (see the text).

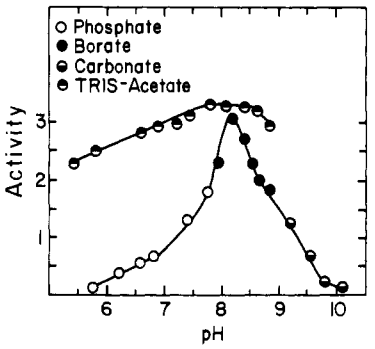

FIGURE 7: pH dependence of the enzyme. The reaction mixtures contained 40 μmoles of buffer (potassium phosphate, sodium borate, sodium carbonate, and Tris-acetate), 4 μmoles of γ-L-glutamyl-γ-L-glutamyl-*p*-nitroanilide, and enzyme (0.03 unit) in a final volume of 0.5 ml.

when γ-glutamyl transpeptidase was present in the reaction mixture (values given in parentheses) the formation of pyrrolidonecarboxylate from almost all of the γ-glutamyl compounds was increased; the values obtained with a number of these were substantially increased, *e.g.*, glutathione, γ-L-glutamyl-*p*-nitroanilide, L-glutamine, γ-L-glutamyl-L-phenylalanine, and γ-L-glutamyl-L-valine. The activity of the enzyme was also tested against α,γ-L-glutamyldiglycine and the reaction was followed by paper chromatography; under conditions similar to those given in Table II, no evidence for formation of free glycine was obtained. The enzymes did not act on α-L-glutamyl-L-alanine, as determined by paper chromatographic procedures capable of detecting glutamate and alanine formation.

Discussion

The procedure described here yields preparations of the enzyme that are considerably more active than those previously described. It is of interest that although the human brain enzyme preparation is apparently homogeneous in the analytical ultracentrifuge, it can be separated into two components possessing different isoelectric points; furthermore, there is evidence for still another active component (Figure 2). The sheep brain enzyme preparation is also heterogeneous when examined by the electrofocusing technique (Figure 6). As far as can be determined from the present results, the several isoenzymes exhibit specific activities of about the same order; however, additional studies must be carried out with larger amounts of material in order to fully investigate this and other properties of these catalytic activities. Studies on the structures of these enzymes may be facilitated by the fact that they are of relatively low molecular weight.

It is notable that the model substrate used here is more active than a large number of other γ-glutamyl compounds that were examined. The relatively high activity of γ-L-glutamyl-γ-L-glutamyl-*p*-nitroanilide and the observation that pyrrolidonecarboxylate formation from many of the γ-glutamyl compounds (including glutathione) is increased considerably when γ-glutamyl transpeptidase is added suggest that the preferred substrates are γ-glutamyl-γ-glutamylamino acids. Thus, the formation of pyrrolidonecarboxylate from glutathione (Woodward and Reinhart, 1942; Fodor *et al.*, 1953) may be explained in terms of reactions A and B.

$$\text{2-glutathione} \xrightarrow[\text{transpeptidase}]{\gamma\text{-glutamyl}} \gamma\text{-glutamylglutathione} + \text{cysteinylglycine} \quad \text{(A)}$$

$$\gamma\text{-glutamylglutathione} \xrightarrow[\text{cyclotransferase}]{\gamma\text{-glutamyl}} \text{pyrrolidonecarboxylic acid} + \text{glutathione} \quad \text{(B)}$$

An analogous explanation may be formulated for the formation of pyrrolidonecarboxylate from other γ-glutamyl compounds. The inactivity of the enzyme toward γ-L-glutamyl-β-alanine, γ-L-glutamyl-β-aminoisobutyric acid, and the three stereoisomers of the model substrate indicates that the enzyme is specific for γ-L-glutamyl derivatives of L-α-amino acids.

The available data show that γ-L-glutamylcyclo-

TABLE II: Specificity of the Enzymes from Human and Sheep Brain.[a]

Substrate	Formation of Pyrrolidonecarboxylate (μmoles/mg min)	
	Human Brain	Sheep Brain
γ-L-Glutamyl-γ-L-glutamyl-*p*-nitroanilide	150 (156)[b]	100 (102)[b]
γ-L-Glutamyl-L-glutamine	64.5 (68.8)	76.5 (81.3)
γ-L-Glutamyl-L-α-alanine	84.7 (77.6)	27.7 (38.8)
γ-L-Glutamylglycine	21.6 (22.5)	9.45 (15.9)
γ-L-Glutamyl-L-α-aminobutyric acid	16.9 (18.5)	
γ-L-Glutamyl-*S*-methyl-L-cysteine	7.3 (15.3)	6.2 (9.45)
γ-L-Glutamyl-L-valine	1.32 (9.7)	0.68 (7.15)
γ-L-Glutamyl-L-leucine	1.28 (7.95)	0.17 (7.15)
γ-L-Glutamyl-L-tyrosine	1.41 (13.5)	0.11 (4.10)
γ-L-Glutamyl-L-phenylalanine	0.75 (8.95)	0.08 (3.10)
γ-L-Glutamylglycine ethyl ester	0.48 (6.75)	0.31 (5.95)
γ-L-Glutamyl-β-aminoisobutyric acid	0.00 (0.31)	0.05 (0.11)
Glutathione (reduced)	0.53 (18.5)	0.28 (17.8)
Glutathione (oxidized)	0 (7.1)	0 (10.5)
γ-L-Glutamyl-β-alanine	0 (0.79)	0 (0.71)
L-Glutamine	0 (2.96)	0 (2.01)
γ-L-Glutamyl-*p*-nitroanilide	0 (59.2)	0 (22.6)
γ-D-Glutamyl-*p*-nitroanilide	0 (0)	0 (0)
γ-D-Glutamyl-γ-D-glutamyl-*p*-nitroanilide	0 (0)	0 (0)
γ-D-Glutamyl-γ-L-glutamyl-*p*-nitroanilide	0 (0)	0 (0)
γ-L-Glutamyl-γ-D-glutamyl-*p*-nitroanilide	0 (4.85)	0 (1.97)

[a] The reaction mixtures contained substrate (4 μmoles; 2 μmoles for γ-L-glutamyl-*p*-nitroanilide), Tris-HCl buffer (pH 8.0, 10 μmoles), and γ-glutamylcyclotransferase (0.1 unit; human brain enzyme specific activity 150 units/mg; sheep brain enzyme specific activity 100 units/mg) in a final volume of 0.25 ml. The mixtures were incubated at 37° for 10–120 min, after which the formation of pyrrolidonecarboxylate was determined as described in the text. [b] The values given in parentheses were obtained in experiments in which 1 unit of γ-glutamyltranspeptidase was added to the reaction mixture.

transferase is widely distributed in animal tissues. As stated above, the enzyme was first found in pig liver (Connell and Hanes, 1956); it was later studied in the subcellular fractions of rabbit liver and found to be present in the supernatant fraction (Cliffe and Waley, 1961). Kakimoto *et al.* (1967) described a 14-fold purification of an enzyme from rat liver acetone powder extract that catalyzes the conversion of γ-L-glutamyl-L-glutamine to pyrrolidonecarboxylic acid and glutamine. This enzyme was reported to be inactive toward γ-glutamylglutamic acid, γ-glutamylglycine, and glutathione, but to exhibit some activity toward γ-glutamylalanine and γ-glutamylserine. These workers also found γ-glutamylglutamine cyclotransferase (lactamase) activity in various organs (kidney, spleen, heart, brain, lung, muscle, intestine, and pancreas) of the rat and guinea pig. Connell and Szewczuk (1967) have reported the presence of γ-glutamylcyclotransferase (γ-glutamyllactamase) in human liver, kidney, serum, and other tissues; these workers used the substrate L-[γ-^{14}C]glutamyl-L-glutamyl-α-naphthylamide and found that the terminal glutamyl group of this compound is converted into [^{14}C]pyrrolidonecarboxylic acid, which was separated by paper electrophoresis and determined by liquid scintillation counting. Studies in our laboratory (B. Smith, 1968, unpublished work), in which the model substrate described here was used, have shown that substantial amounts of the enzyme are present in the skin, testes, kidney, brain, heart, liver, spleen, muscle, intestine, and lung of the mouse and guinea pig.

The wide distribution of γ-glutamylcyclotransferase activity suggests that this enzyme could be of metabolic significance in many mammalian tissues. However, the physiological role of the enzyme as well as of a number of γ-glutamylamino acids that have been found in nature remains to be elucidated. The enzyme could participate in the metabolism of glutathione as stated above in those tissues which also possess γ-glutamyl transpeptidase (*e.g.*, kidney, pancreas, liver, spleen, and brain). In relation to the possible metabolic functions of γ-glutamylcyclotransferase, we have considered the idea that a γ-glutamyl moiety might be the initial N-terminal residue formed in protein synthesis, and that it might be cleaved by γ-glutamylcyclotransferase after completion of the peptide chain. We have also considered the possibility that γ-glutamylcyclotransferase and γ-glutamyl transpeptidase function in the transport of amino acids in the kidney. γ-Glutamyltranspeptidase is associated with cell membrane material in the kidney while γ-glutamylcyclotransferase is found in the soluble cytoplasmic fraction. Thus, a

γ-glutamyl transpeptidase catalyzed reaction between glutathione and free amino acids might be associated with entrance of free amino acids into the cell in the form of γ-glutamylamino acids, which serve as substrates for γ-glutamylcyclotransferase, thus yielding pyrrolidonecarboxylate and free intracellular amino acids. The reported occurrence of γ-glutamyl linkages in collagen (Gallop *et al.*, 1960) and data available on the metabolism of pyrrolidonecarboxylate (Niwaguchi *et al.*, 1965) represent additional areas of study which may offer clues as to the metabolic function of γ-glutamylcyclotransferase.

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