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Purification and Characterization of the 4-Aminobutyrate–2-Ketoglutarate Transaminase from Mouse Brain†

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ABSTRACT: 4-Aminobutyrate-2-ketoglutarate transaminase (EC 2.6.1.19) from mouse brain has been purified 1200-fold over a brain homogenate using ammonium sulfate fractionation and various column procedures. The specific activity of the purified enzyme was 5.0 units/mg and it appeared homogeneous in polyacrylamide gel electrophoresis and high speed sedimentation equilibrium ultracentrifuge runs from which a partial specific volume of 0.742 and a molecular weight of 109,000 were calculated. The pH optimum for the enzyme was 8.05 and the K_m values for γ-aminobutyrate and α-ketoglutarate were estimated to be 1.1 and 0.25 mM, respectively. Of the keto acids tested only α-ketoglutarate was an amino group acceptor. However, of a series of amino acids tested, β-alanine, δ-aminovaleric acid, and β-aminoisobutyric acid were effective amino donors in the reaction catalyzed by the enzyme.

During recent years much information has accumulated concerning the dual function of γ-aminobutyric acid as a metabolite and inhibitory transmitter in invertebrate peripheral nerves and the vertebrate central nervous system (Otsuka *et al.*, 1966; Krnjevic and Schwartz, 1967; Roberts and Kuriyama, 1968; Baxter, 1970). Accordingly it has become increasingly important to obtain more detailed knowledge about the two enzymes directly involved in the metabolism of γ-aminobutyric acid, *i.e.*, the glutamic acid decarboxylase (EC 4.1.1.15) and the 4-aminobutyrate-2-ketoglutarate transaminase (EC 2.6.1.19). Although the latter enzyme has been purified from brain to some extent before (Waksman and Roberts, 1965; Sytinsky and Vasilijev, 1970) little is known with certainty about its properties. The decarboxylase has recently been obtained as a homogeneous protein in this laboratory (Wu *et al.*, 1973) and it seemed therefore appropriate also to undertake the purification of the transaminase.

Furthermore, a highly purified enzyme is a prerequisite for the production of a pure antibody by which it may be possible to localize the enzyme in the brain by the immunohistochemical technique described by Nakane and Pierce (1967). This method might give a specific and precise localization of the transaminase, which is essential to fully understand the mechanism of γ-aminobutyric acid as a neurotransmitter.

This article describes the purification procedures and some properties of the transaminase from mouse brain.

Experimental Procedures

Materials

Whole brains from Swiss albino mice were used as the source for the enzyme. The mice were obtained from Horton Laboratories (Oakland, Calif.). Ammonium sulfate was special enzyme grade from Schwarz/Mann (Orangeburg, N. Y.). 3-Acetylpyridine-NAD$^+$ was purchased from P-L Biochemicals (Milwaukee, Wis.) and glutamate dehydrogenase, type II, and aldehyde dehydrogenase from Sigma Chemical Co. (St. Louis, Mo.). Sephadex G-200 and DEAE-Sephadex were purchased from Pharmacia Fine Chemicals (Piscataway, N. J.) and calcium phosphate gel from Bio-Rad Laboratories (Richmond, Calif.). Pyridoxal phosphate was donated by Calbiochem (La Jolla, Calif.). All other chemicals were of purest grade available from regular commercial sources.

Methods

Standard Activity Assay. A widely used method for determination of γ-aminobutyrate transaminase activity is the radioassay (*cf.* Waksman and Roberts, 1963) in which ^{14}C-labeled α-ketoglutarate and cold γ-aminobutyrate are used as the substrates. The latter method is useful for measuring low activities because of its sensitivity, but it is impractical to employ as a standard assay method in purification procedures during which many column fractions must be assayed. A rapid spectrophotometric method was developed which in principle is similar to previously used methods (*cf.* Baxter, 1970).

The transaminase reaction was performed in a 100 mM Tris-HCl buffer, pH 8.0, containing 20 μM pyridoxal phosphate, 100 μM AET,[1] 50 mM γ-aminobutyric acid, and 10 mM α-ketoglutaric acid. This buffer plus enzyme was incubated in a water bath with shaking for 30 min at 37°. Under the above conditions the reaction is linear with time for 1 hr. The reaction was stopped by the addition of aminooxyacetic acid to a final concentration of 100 μM, and the tubes were immediately transferred to an ice bath. Aminooxyacetic acid is known to be a very potent inhibitor of the γ-aminobutyric acid transaminase (Wallach, 1961). Blanks run either without enzyme or

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[1] Abbreviation used is: 2-aminoethylisothiouronium bromide hydrobromide, AET.

in the presence of 100 μM aminooxyacetic acid gave comparable low values in the subsequent determination of glutamate.

The glutamate formed in the transaminase reaction was determined in aliquots of the reaction mixture using glutamate dehydrogenase and acetylpyridine-NAD$^+$ to catalyze the oxidation of glutamate to α-ketoglutarate and measuring the increase in optical density at 363 nm attributable to acetylpyridine-NADH in a Zeiss spectrophotometer. The buffer for this reaction had the following composition: 100 mM Tris-HCl, pH 8.0, 25 mM hydrazine, 1 mM aminooxyacetic acid, 750 μM acetylpyridine-NAD$^+$, and glutamate dehydrogenase (1.3 units/ml). The reaction was carried out at room temperature for 90 min. Acetylpyridine-NAD$^+$ was used instead of NAD$^+$ because it increases the equilibrium constant of the reaction by a factor of 100 (Kaplan *et al.*, 1956; Holzer and Soling, 1962) and the reduced form has a 50% higher extinction coefficient than does NADH. Aminooxyacetic acid and hydrazine which are carbonyl trapping agents were included to trap the α-ketoglutarate formed to ensure a completion of the reaction. Under these conditions the conversion of glutamate to α-ketoglutarate was in excess of 95%.

The enzyme activity is expressed as units/milliliter of enzyme solution and specific activities (sp act.) as units/milligram of protein. One unit is defined as that activity catalyzing the formation of 1 μmol of glutamate/min at 37°.

Protein Determination. Proteins were measured using the method described by Lowry *et al.* (1951) with the modifications proposed by Miller (1959). Bovine serum albumin was used as the standard.

Effect of pH. The standard assay was used substituting other buffers for Tris-HCl buffer. A phosphate buffer (100 mM) was used from pH 6.5 to 7.5, Tris-HCl (100 mM) was used from pH 8.0 to 8.5, and bicarbonate buffer (100 mM) was used for pH between 8.5 and 9.5.

Determination of K_m Values for γ-Aminobutyrate and α-Ketoglutarate. Because the activity of glutamate dehydrogenase is dependent upon the concentration of α-ketoglutarate, an alternative assay method was used to determine initial velocities of the transaminase reaction at different substrate concentrations (γ-aminobutyrate, 0.1–4.0 mM, α-ketoglutarate, 0.04–1.0 mM). It was recently reported (Nikolaeva and Vasilijev, 1972) that an unspecific aldehyde dehydrogenase from yeast (Lundquist, 1958) will oxidize succinic semialdehyde to succinic acid using NAD$^+$ as the coenzyme. The reaction buffer had the following composition: 100 mM Tris-HCl, pH 8.0; 10 mM KCl; 1 mM β-mercaptoethanol; 100 μM AET; 20 μM pyridoxal phosphate; 300 μM NAD$^+$; and 1 unit/ml of aldehyde dehydrogenase. Different amounts of γ-aminobutyrate and γ-ketoglutarate were added and the reaction was started by addition of the purified transaminase (sp act. 5.0 units/mg) and followed at 340 nm for approximately 10 min using a Gilford automatic multiple sample recorder attached to a Beckman DU spectrophotometer. The initial velocities obtained in this way at different substrate concentrations were used for double reciprocal plots and a set of parallel lines for each substrate was obtained for a series of fixed concentrations of the other substrate. From these data the apparent K_m values could be obtained (Velick and Vavra, 1962).

Substrate Specificity. The specificity of the enzyme for the amino acid was studied employing the standard assay where the amino acid in question was substituted for γ-aminobutyric acid, usually at the same concentration (50 mM). Because of low solubilities tyrosine and tryptophan were used at lower concentrations (2 and 10 mM, respectively). To determine specificity for the keto acid the assay employing aldehyde dehydrogenase (*cf.* determination of K_m) was used. α-Ketoglutaric acid was replaced by different mono- and dicarboxylic keto acids (10 mM) and γ-aminobutyric acid was used as the other substrate.

Polyacrylamide Gel Electrophoresis. Polyacrylamide electrophoresis was performed according to the original procedure of Davis (1964). The sample was applied on a 5% polyacrylamide separating gel column (0.5 × 8 cm) containing 0.184% *N,N'*-ethylenebisacrylamide, 0.0625% 3-dimethylaminopropionitrile, 0.75 M Tris, and 0.12 M HCl (pH 8.9). Ammonium persulfate was used as the polymerizing agent. A Tris–glycine buffer (11.0 g of Tris and 14.4 g of glycine per l.), pH 9.3, was used as the electrophoresis buffer. Electrophoresis was carried out at 4° for 90 min using a current of 3.0 mA/column. The samples consisted of 20–40 μg of the most purified enzyme in 20–40 μl of 20% sucrose containing 0.01% Bromophenol Blue to mark the front.

For protein staining gels were placed in 0.5% Amido Black 10B in 7% acetic acid at room temperature for 1 hr after electrophoresis and then electrophoretically destained in 7% acetic acid using a Canalco quick gel destainer. For enzyme assay immediately after electrophoresis the gels were cut into 5 mm slices. These were homogenized individually in 0.5 ml of transaminase reaction buffer (*cf.* standard assay) at 0–4°. The tubes then were incubated at 37° for 1 hr, the reaction was stopped by the addition of aminooxyacetic acid, the gel pieces were removed by filtration, and an aliquot of the filtrate was used for glutamate determination as described above.

Determination of Molecular Weight and Partial Specific Volume. The apparent molecular weight and the partial specific volume of the transaminase were determined simultaneously by measuring sedimentation equilibrium in two different solvents as described by Edelstein and Schachman (1967).

The high speed sedimentation equilibrium method of Yphantis (1964) was employed. Experiments were carried out at 7° with Yphantis' six-channel rectangular cell in a Spinco Model E analytical ultracentrifuge equipped with Rayleigh interference optics. Runs were continued until no further change in the distribution of concentration in the cell occurred. Sapphire windows were used to minimize the optical distortion. Plates were analyzed in a Nikon microcomparator with 50-fold magnification. Samples in D_2O were prepared by dialyzing 1 ml of enzyme solution in standard buffer against 100 ml of D_2O buffer solution with three changes overnight. The densities of the solutions were determined with a pycnometer.

Purification of γ-Aminobutyric Acid Transaminase. INITIAL STEPS. The initial steps in the procedure for the preparation of starting material for the purification of the γ-aminobutyric acid transaminase were identical with those employed in the purification of glutamic acid decarboxylase from mouse brain by Wu *et al.* (1973). These are the homogenization of the tissue in 0.25 M sucrose and the subsequent centrifugations which finally gave a fraction called the crude mitochondrial fraction. After liberation of the decarboxylase from the latter by hypotonic shock and subsequent centrifugation for 75 min at 105,000*g* in a Spinco L-2 centrifuge, the pellet was used as the source for the transaminase. This pellet, representing 300 brains, was suspended in 150 ml of water containing 1.0 mM AET and 0.2 mM pyridoxal phosphate, pH 7.2, disrupted in a Waring Blendor and centrifuged for 75 min at 105,000*g*. All operations were carried out at 4° unless otherwise mentioned. The supernatant was collected and concentrated potassium

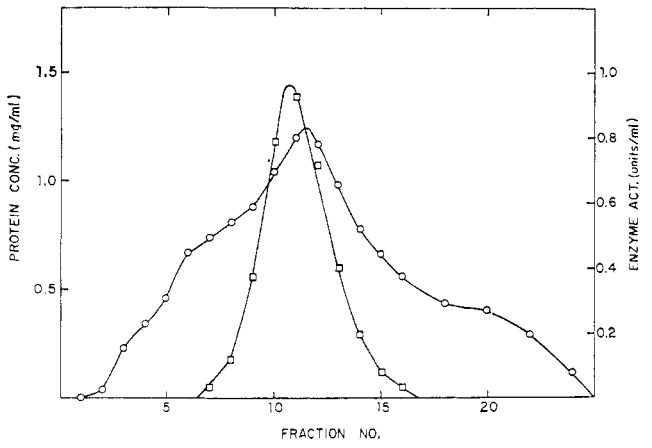

FIGURE 1: Gel filtration of the transaminase from the ammonuim sulfate fractionation on Sephadex G-200. The protein was applied and eluted with 0.05 M potassium phosphate, pH 7.2. Protein concentration (○) is given in mg/ml and enzyme activity (□) in units/ml; 13 ml was collected in each fraction.

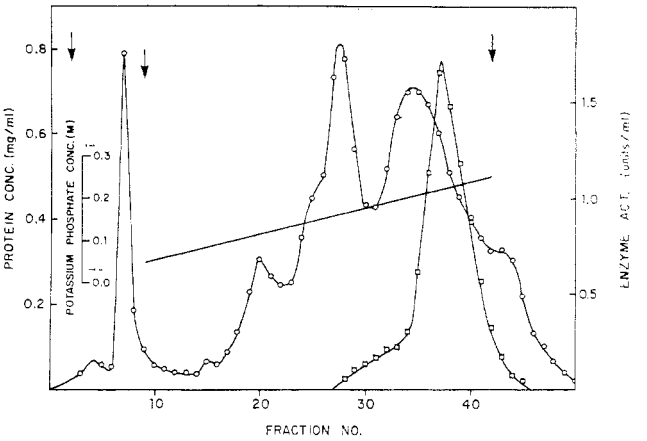

FIGURE 2: Chromatography on calcium phosphate gel of the concentrated peak fractions from Sephadex G-200 columns. The protein was applied in 0.010 M potassium phosphate, pH 7.2, and washed with 0.05 M potasium phosphate, pH 7.2, starting at the first arrow. The second arrow indicates the start of a linear gradient of potassium phosphate, pH 7.2, from 0.05 to 0.25 M. The third arrow indicates that the elution is continued with 0.25 M potassium phosphate, pH 7.2: (○) protein concentration (mg/ml); (□) enzyme activity (units/ml); (—) theoretical concentration gradient (*M*). Each fraction represents 13 ml of eluate.

phosphate buffer, pH 7.2, was added to give a final phosphate concentration of 0.05 M. Also, EDTA was added to a final concentration of 0.1 mM. About 24% of the total enzyme activity in the original homogenate was recovered in this crude mitochondrial extract with a 34-fold purification. This extract was stable for several months at −20°.

AMMONIUM SULFATE FRACTIONATION. Four batches of the mitochondrial extract were thawed overnight at 4° and solid ammonium sulfate was added to give 40% saturation (243 g/l.). The pH was maintained at 7.2 by the addition of 0.1 M NH_4OH (0.5 ml/g of ammonium sulfate). After 20 min of stirring it was centrifuged in a Sorvall RC2-B refrigerated centrifuge at 14,000*g* for 30 min. The pellet was discarded and the supernatant made 80% saturated with ammonium sulfate 285 g/l.), again maintaining the pH at 7.2 by the addition of NH_4OH. This was stirred and centrifuged as described above and the supernatant discarded. The pellet was dissolved in 0.05 M potassium phosphate, pH 7.2, containing 1.0 mM AET, 0.2 mM pyridoxal phosphate, and 0.1 mM EDTA, and centrifuged at 14,000*g* for 30 min (Sorvall RC2-B) in order to remove insoluble material. All buffer solutions contained 1.0 mM AET, 0.2 mM pyridoxal phosphate, and 0.1 mM EDTA unless otherwise mentioned. The supernatant was applied directly to a Sephadex G-200 column (*cf.* below) or if storage was desired dialyzed overnight against three changes of large volumes of 0.05 M potassium phosphate buffer, pH 7.2, and subsequently frozen and kept at −20°. The recovery of enzyme activity in the ammonium sulfate fraction (40–80%) was about 85% with a twofold purification.

GEL FILTRATION. A column, 2.5 × 100 cm (Pharmacia, Sweden), was packed with Sephadex G-200 treated according to the manufacturer's recommendations. After packing, the column was equilibrated with 0.05 M potassium phosphate, pH 7.2, prior to the application of the dissolved pellet from the 80% ammonium sulfate precipitation (*cf.* above). The protein was eluted with the same buffer as used for the equilibration and a typical elution profile is shown in Figure 1. The recovery of enzyme activity was 100% and the purification at the peak fraction was two- to threefold. The fractions containing the enzyme activity were divided into three batches with specific activities between 0.5 and 0.8 unit/mg, 0.25 and 0.5 unit/mg, and 0.08 and 0.25 unit/mg, respectively. The highest specific activity fractions from four columns were pooled and concentrated by the addition of solid ammonium sulfate to 85% saturation (610 g/l.). The pH was maintained at 7.2 by the addition of 0.1 M NH_4OH. After 20 min of stirring and subsequent centrifugation (14,000*g* for 30 min in a Sorvall RC2-B), the pellet was dissolved in 0.05 M potassium phosphate, pH 7.2, and this was used for the next purification step (*cf.* below). The intermediate fractions were pooled and concentrated in the same way and then reapplied to a Sephadex G-200 column. The fractions with the lowest specific activities were treated in the same way as the crude mitochondrial extracts.

CALCIUM PHOSPHATE GEL CHROMATOGRAPHY. A column, 2.5 × 20 cm (Pharmacia, Sweden), was packed with calcium phosphate gel prepared as described by Tiselius *et al.* (1956) and equilibrated with 0.010 M potassium phosphate, pH 7.2. The dissolved pellet from the ammonium sulfate precipitation of the high specific activity fractions from the Sephadex G-200 column was dialyzed overnight against three changes of large volumes of 0.010 M potassium phosphate, pH 7.2. After centrifugation (14,000*g;* 30 min in a Sorvall RC2-B), the supernatant was applied to the equilibrated column, and an equal volume of 0.010 M potassium phosphate, pH 7.2, was introduced. The column was then washed with approximately 100 ml of 0.05 M potassium phosphate, pH 7.2, and after this washing step the enzyme was eluted using a linear concentration gradient of potassium phosphate, pH 7.2, from 0.05 to 0.25 M. After the gradient was finished the elution was continued with 0.25 M potassium phosphate, pH 7.2. A typical elution profile is shown in Figure 2. The enzyme is eluted at phosphate concentrations from 0.17 to 0.25 M with the peak fraction appearing at 0.22 M. The ionic strength required to elute the enzyme depends upon the ratio between the amount of protein and the amount of gel. The less gel the lower the phosphate concentration needed. The recovery of enzyme activity was about 95% with a four- to fivefold purification at the enzyme peak. The fractions containing the enzyme activity were again divided into three batches with specific activities between 1.7 and 3.2 units/mg, 0.5 and 1.7 units/mg, and lower than 0.5 unit/mg. The intermediate fractions were

TABLE I: Purification of γ-Aminobutyric Acid Transaminase from Mouse Brain.[a]

Sample	Vol (ml)	Total Protein (mg)	Total Act. (Units)	Sp Act. (Units/mg) × 10^2	Yield (%) (Act.)	Purification
Sucrose homogenate	30,000	480,000	2,000	0.417	100	1.0
Crude mitochondrial extract	4,950	3,300	475	14.2	23.8	34.1
1st $(NH_4)_2SO_4$ (40–80%)	150	1,350	400	29.7	20.0	71.5
Sephadex G-200 (pool)	400	500	317	63.4[b]	15.8	152
2nd $(NH_4)_2SO_4$ (0–85%)	50	425	268	63.4	13.4	152
Calcium phosphate gel (pool)	130	80	200	250[c]	10.0	600
3rd $(NH_4)_2SO_4$ (0–85%)	8	75	188	250	9.4	600
DEAE-Sephadex (pool)	80	21	105	500	5.3	1200
Ultrafiltration	5	20	100	500	5.0	1200

[a] The purification was made from 9000 brains. [b] The peak fraction had a sp act. of 75.0. [c] The peak fraction had a sp act. of 300.

pooled, concentrated with ammonium sulfate (85% saturation), and reapplied to the calcium phosphate column which could be regenerated by wash with 0.5 M potassium phosphate, pH 7.2, without AET, pyridoxal phosphate, and EDTA and subsequent equilibration with 0.010 M potassium phosphate, pH 7.2. Low specific activity fractions (sp act. <0.5 unit/mg) were treated in the same way as the intermediate specific activity fractions from the Sephadex G-200 columns. The highest specific activity fractions from three columns were pooled and the protein precipitated with ammonium sulfate (85% saturation). After centrifugation (14,000*g*; 30 min in a Sorvall RC2-B) the pellet was dissolved in 0.05 M potassium phosphate, pH 7.2, and dialyzed overnight against three changes of the same buffer. After centrifugation (14,000*g*; 30 min in a Sorvall RC2-B) the supernatant was used in the last step of the purification.

DEAE-SEPHADEX CHROMATOGRAPHY. A column, 2.5 × 50 cm (Pharmacia, Sweden), was packed with DEAE-Sephadex, A-50 beaded form, previously equilibrated with 0.05 M potassium phosphate, pH 7.2. After packing, the column was equilibrated with the same buffer and the concentrated high specific activity fractions from the preceding step were applied to the column. After the application the column was washed with the same buffer (150 ml) and the enzyme was eluted with a linear concentration gradient of potassium phosphate, pH 7.2, from 0.05 to 0.15 M. The enzyme started to appear at 0.12 M potassium phosphate and the peak fraction corresponded to 0.15 M potassium phosphate (Figure 3). The purification was twofold and the recovery of enzyme activity about 95%. The protein peak and the enzyme peak coincided giving several fractions with almost identical specific activities. These fractions were pooled and concentrated using a high pressure ultrafiltration device (Creative Scientific Equipment, Corp.) with an Amicon membrane filter PM 30 which retains molecules with molecular weights higher than 30,000. Approximately 95% of the enzyme activity was recovered. The concentrated enzyme solution was divided into small aliquots which were kept frozen in the dark at −20°.

Results

Purification. The purification procedure is summarized in Table I. The enzyme was purified 1200 times over the brain homogenate and the sp act. was 5.0 units/mg of protein. The recovery of enzyme activity was about 5%. The purified enzyme protected with AET and pyridoxal phosphate is stable at −20° in the dark for several months. Thawing and freezing cause a slight decrease in activity.

Criteria of Purity. (a) Polyacrylamide gel electrophoresis of 20–40 μg of the purified enzyme revealed a single protein band with the location of the enzyme activity corresponding to the location of the protein band (Figure 4).

(b) The fraction representing a 1200-fold purification over the homogenate appeared to be homogeneous in size as judged from the linear plot of the logarithm of concentrations, *c*, against the squares of the distances, *r*, from the center of rotation to points of interest in high speed sedimentation equilibrium runs in both H_2O and D_2O solutions.

Molecular Weight. The partial specific volume was calculated from the slopes obtained from the plots of log *c* (in terms of fringe displacements in microns) *vs.* r^2 in square centimeters. The density measurements indicated the presence of 93% D_2O, which by interpolation (κ = 1.0155 in a 100% D_2O solution) gave a κ value of 1.0144. This gave a value of 0.742 for the partial specific volume of the transaminase.

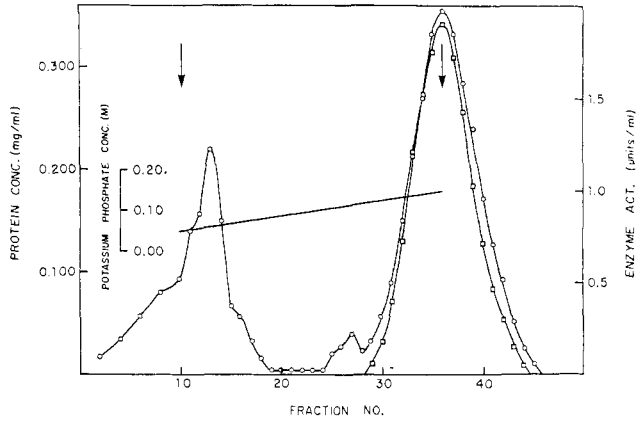

FIGURE 3: Chromatography on DEAE-Sephadex of the concentrated peak fractions from calcium phosphate columns. The protein was applied in 0.05 M potassium phosphate, pH 7.2, and washed with the same buffer. The first arrow indicates the start of linear gradient of potassium phosphate, pH 7.2, from 0.05 to 0.15 M. The second arrow indicates that the elution is continued using 0.15 M potassium phosphate, pH 7.2: (○) protein concentration (mg/ml); (□) enzyme activity (units/ml); (—) theoretical concentration gradient (M). The volume of the fractions was 13 ml.

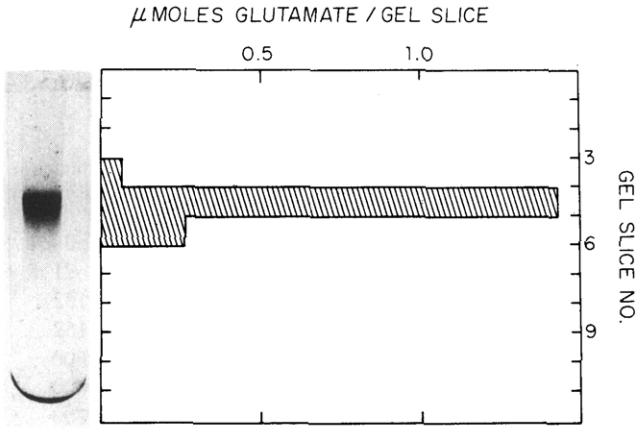

FIGURE 4: Gel electrophoresis on a 5% polyacrylamide separating gel column of 25 μg of the most highly purified protein (sp act. 5.0 units/mg). The protein pattern is shown on the left side and the migration is from top (cathode) to bottom (anode). The electrophoresis buffer was Tris-glycine, pH 9.3, and the current 3 mA/column. Transaminase activity measured in slices of a parallel gel is given in the right side in terms of μmol of glutamate formed/gel slice (5 mm).

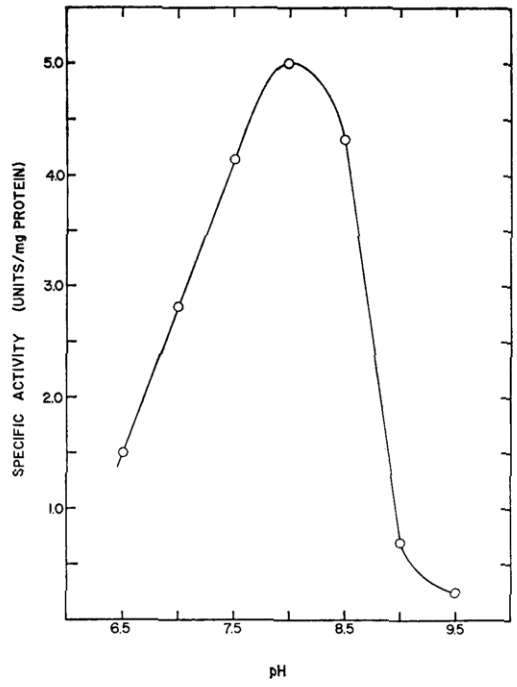

FIGURE 5: Specific activity of the purified enzyme (units/mg) as a function of pH in the incubation medium. The standard assay (*cf.* Experimental Procedures) was employed and phosphate buffers (pH 6.5–7.5), Tris-HCl buffers (pH 8.0–8.5), or bicarbonate buffers (pH 9.0–9.5) were used to obtain the desired pH.

We could then determine the apparent molecular weight, which was virtually concentration independent in the range of concentrations used (0.1–0.3 mg/ml). Thus, we obtained a weight average mol wt of 109,000 for the transaminase at infinite dilution.

Effect of Protein Concentration on Enzyme Activity. The enzyme activity was found to be proportional with the protein concentration over the range of 0–100 μg/ml. This, in turn, means that the specific activity of the transaminase is independent of the protein concentration within the range tested.

Substrate Specificity. None of the following L-α-amino acids gave any activity when substituted for γ-aminobutyric acid in the assay system employing the most highly purified enzyme preparation: glycine, alanine, valine, leucine, isoleucine, proline, histidine, ornithine, lysine, arginine, aspartic acid, asparagine, serine, threonine, methionine, cysteine, tryptophan, phenylalanine, and tyrosine. In addition some amino acids containing amino groups in other than the α position were tested. The results are summarized in Table II. β-Alanine is the only other amino acid which is transaminated to the same extent as γ-aminobutyric acid. ω-Amino acids with carbon chains longer than γ-aminobutyric acid were found to be poor substrates and no activity was observed when more than five carbon atoms were interposed between the amino group and the carboxyl group. β-Aminoisobutyric acid was a relatively good substrate and β-hydroxy-γ-aminobutyric acid was transaminated only to a slight extent under the assay conditions. It was also shown that the β-alanine analogs, taurine and 2-aminoethylphosphonic acid ($H_2N(CH_2)_2H_2PO_3$), and the γ-aminobutyric acid analog, 3-aminopropylphosphonic acid ($H_2N(CH_2)_3H_2PO_3$), could not be transaminated by the purified enzyme.

When α-ketoglutarate was replaced by isomolar amounts of oxaloacetate, α-ketoadipate, pyruvate, α-ketobutyrate, or phenylpyruvate no enzyme activity was observed.

Effect of pH on the Reaction. The specific activity of the transaminase as a function of pH is shown in Figure 5. The pH optimum for the enzyme was found to be between 8.0 and 8.1.

Determination of K_m Values for γ-Aminobutyric Acid and α-Ketoglutaric Acid. Double reciprocal plots of initial velocity against the concentration of one substrate (s_1) at a series of fixed concentrations of the other substrate (s_2) (*cf.* Experimental Procedures) gave a set of parallel lines. The reciprocal K_m values for s_1, obtained from these parallel lines, were plotted against the reciprocal concentrations of s_2 and *vice versa*. These straight lines give at the intercepts of the ordinates the apparent reciprocal K_m value for one substrate at infinite concentration of the other substrate. The K_m value for γ-aminobutyric acid was calculated to be 1.1 mM and for α-ketoglutaric acid, 0.25 mM.

TABLE II: Substrate Specificity of the γ-Aminobutyrate–α-Ketoglutarate Transaminase.

Amino Group Donor	Rel Act. (% of γ-Aminobutyrate)
Glycine	0
β-Alanine	100
DL-β-Aminoisobutyric acid	55
DL-β-Aminobutyric acid	0
β-Aminoglutaric acid	0
γ-Aminobutyric acid	100
DL-β-Hydroxy-γ-aminobutyric acid	5
L-α,γ-Diaminobutyric acid	0
DL-α,γ-Diaminoglutaric acid	0
δ-Aminovaleric acid	48
ε-Aminocaproic acid	6
ω-Aminoheptanoic acid	0

Discussion

The methods employed in this report for the purification of the γ-aminobutyric acid transaminase were highly reproducible. The use of a crude mitochondrial extract in which only 25% of the total enzyme activity in whole brain homogenate is recovered represented a very powerful purification step (34-fold purification) and allowed us to use the same mouse brains that were employed for the purification of the glutamic acid decarboxylase (Wu *et al.*, 1973).

This transaminase has previously been purified 150-fold from mouse brain (Waksman and Roberts, 1965) and 400-fold from rat brain (Sytinsky and Vasilijev, 1970). Accordingly, the present preparaton represents the most highly purified γ-aminobutyric acid transaminase ever obtained from mammalian brain since it has been purified 1200-fold based upon the specific activity of the original brain homogenate, which is in good agreement with a value previously given by Waksman *et al.* (1968).

The most highly purified enzyme obtained in this study was found to be homogeneous as judged from the following findings. In the last purification step the protein peak closely coincided with the enzyme peak yielding several fractions with essentially identical specific activities. Only one protein band was seen after polyacrylamide electrophoresis, and this protein band coincided with all of the enzyme activity observed after the slicing of a duplicate gel. The results from the high speed sedimentation equilibrium strongly suggest that the enzyme is homogeneous in terms of molecular size, within the limits of the method employed. None of the 19 naturally occurring α-amino acids tested could serve as the amino group donor in the transaminase reaction catalyzed by the most highly purified enzyme. It is especially notable that no activity was found with aspartic acid, since the activity of the aspartic acid transaminase is more than a 100-fold higher than that of γ-aminobutyric acid transaminase in brain homogenate (Benuck *et al.*, 1972). β-Alanine and δ-aminovaleric acid were effective amino donors. That these reactions are due to the γ-aminobutyric acid transaminase is strongly supported by the finding that these amino acids showed the same relative reactivities in a crude enzyme preparation (Baxter and Roberts, 1958). From the data in Table II and the finding that the analogs of β-alanine and γ-aminobutyric acid could not be transaminated it appears that the amino donor must be a neutral ω-aminocarboxylic acid with the two functional groups being separated by from two to four carbon atoms. Substitutions in the carbon chain strongly impaired the reactivity of the compound. However, of the α-keto acids tested only α-ketoglutaric acid appeared to be an effective substrate.

The basic kinetic properties of the purified transaminase obtained in this study are somewhat different from those previously reported for partially purified preparations (Waksman and Roberts, 1965; Sytinsky and Vasilijev, 1970). The specific activity of the enzyme is independent of the protein concentration and the apparent K_m values for γ-aminobutyric acid and α-ketoglutaric acid are lower than those previously reported. The pH optimum, however, is similar to that reported by Waksman and Roberts (1965). The relatively low value of the Michaelis constant found for γ-aminobutyric acid indicates that the rate of the transaminase reaction *in vivo* in brain is not limited by the concentration of this substrate which presumably is higher than the K_m value in most brain regions (Baxter, 1970). The concentration of α-ketoglutaric acid in brain is, however, probably somewhat lower than the K_m value for this substrate (Cheng, 1971). This might be of importance for the activity of the enzyme *in vivo*. Thus, α-ketoglutaric acid might play an important role in the regulation of the overall γ-aminobutyric acid level in the brain since it has been reported (Wu, 1972) that α-ketoglutaric acid is a strong inhibitor of the glutamic acid decarboxylase, which is the enzyme responsible for the synthesis of γ-aminobutyric acid.

The finding that this enzyme has a mol wt of 109,000 suggests that it is likely to contain more than one polypeptide chain. This, in turn, means that the enzyme may consist of subunits, and this possibility is now under investigation.

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