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Purification and characterization of cysteic acid and cysteine sulfinic acid decarboxylase and L-glutamate decarboxylase from bovine brain

(taurine/amino acid transmitters/γ-aminobutyric acid)

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ABSTRACT L-Cysteic and cysteine sulfinic acids decarboxylase (CADCase/CSADCase) and L-glutamic acid decarboxylase (GADCase), the synthetic enzymes for taurine and γ-aminobutyric acid, respectively, have been purified to homogeneity from bovine brain. Although CADCase/CSADCase and GADCase copurified through various column procedures, these two enzymes can be clearly separated by a hydroxyapatite column. The purification procedures involve ammonium sulfate fractionation, column chromatographies on Sephadex G-200, hydroxyapatite, DEAE-cellulose, and preparative polyacrylamide gel electrophoresis. The K_m values for CADCase/CSADCase are 0.22 and 0.18 mM with L-cysteic and cysteine sulfinic acids as substrates, respectively. CADCase/CSADCase cannot use L-glutamate as substrate. GADCase can use L-glutamate, L-cysteic, and cysteine sulfinic acid as substrates with K_m values of 1.6, 5.4, and 5.2 mM, respectively. Antibodies against CADCase/CSADCase do not cross-react with GADCase preparations and vice versa. It is concluded that CADCase/CSADCase and GADCase are two distinct enzyme entities and they are responsible for the biosynthesis of taurine and γ-aminobutyric acid, respectively.

Taurine and γ-aminobutyric acid (GABA) are two structurally related amino acids and both have been proposed as inhibitory neurotransmitters or modulators in the mammalian central nervous system (for review, see refs. 1–3). The GABA-synthesizing enzyme, L-glutamate decarboxylase (GADCase; EC 4.1.1.15), has been purified from mouse brain and catfish brain (4, 5) and GADCase-specific antibodies have also been obtained (4, 6). Furthermore, the GABA-containing neurons and the GABA-ergic pathways have also been extensively studied by immunocytochemical methods (for review, see refs. 7 and 8). Unlike the GABA system, the biosynthetic pathways for taurine in the mammalian brain are not clear. It has been postulated that the major route for taurine synthesis in brain is through the decarboxylation of cysteine sulfinic acid to hypotaurine by cysteine sulfinic acid decarboxylase (CSADCase; EC 4.1.1.29) and the subsequent oxidation of hypotaurine to taurine (9). An alternative pathway is the oxidation of cysteine sulfinic acid to cysteic acid, followed by the decarboxylation of cysteic acid to taurine by cysteic acid decarboxylase (CADCase) (9). Although the nature of the enzyme(s) decarboxylating cysteine sulfinic and cysteic acids is still disputed (10, 11), the balance of the evidence indicates that the decarboxylation of cysteine sulfinic and cysteic acids is catalyzed by the same enzyme (9, 12–14). Recently, a controversy arose as to whether the same enzyme may catalyze the decarboxylation of L-glutamate and L-cysteine sulfinate in rat brain (15, 16). This view is supported by the observations that GADCase purified from both rat and human brains is capable of decarboxylating both L-glutamate and L-cysteine sulfinate (15, 16). Furthermore, kinetic studies on enzymatic activities by cross-incubation tests of Dixon and Webb (17) also excluded the existence of two enzymes (15, 16). Because GADCase is the rate-limiting enzyme for GABA biosynthesis, its presence in certain types of neurons has been regarded as evidence to support the notion that these neurons use GABA as their neurotransmitter. Should GADCase and CSADCase/CADCase be the same enzyme entity, the GABA neurons identified by the presence of GADCase will actually include the CSADCase/CADCase-containing neurons.

This communication describes the purification of GADCase, CADCase, and CSADCase from bovine brain and presents evidence to show that different enzyme entities are responsible for the biosynthesis of GABA and taurine in bovine brain.

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MATERIALS AND METHODS

Preparation of [^{35}S]Cysteic Acid. Cysteic acid was synthesized by a modification of performic acid oxidation of cystine described by Moore (18) and purified by anion exchange column chromatography as described by Wu *et al.* (19).

Enzyme Assays. The assay of CADCase was based on the formation of [^{35}S]taurine from [^{35}S]cysteic acid and the separation of taurine from cysteic acid by a rapid filtration–ion exchange resin method as described by Wu *et al.* (19). GADCase was assayed either by the CO_2 method as described by Wu (4) or the GABA method as described by Chude and Wu (20). The conditions and the procedures for assaying CSADCase were the same as those employed for CADCase except that $^{14}CO_2$ derived from L-[1-^{14}C]cysteine sulfinic acid (Research Products International) was measured by the CO_2 method as described for GADCase (4).

Electrophoresis. Polyacrylamide slab gel electrophoresis was carried out according to the procedure described by Gabriel (21) and with a device similar to that described by Amos (22). For preparative gel electrophoresis, 500 μl of the concentrated enzyme solution was applied to a 7% polyacrylamide slab gel. The enzyme solution was in 10% glycerol containing bromphenol blue as marker. Before application of the sample, a current of 20 mA was passed through the gel for 30 min in buffer containing 25 mM Tris, 192 mM glycine, 0.065 mM reduced glutathione, 0.2 mM pyridoxal phosphate, and 0.1% 2-mercaptoethanol (pH 8.4). Electrophoresis was carried out at 4°C at 15 mA for the first hour and at 20 mA for an additional hour with the same buffer system as described except that no 2-mercap-

Abbreviations: GABA, γ-aminobutyric acid; GADCase, L-glutamic acid decarboxylase; CADCase, L-cysteic acid decarboxylase; CSADCase, L-cysteine sulfinic acid decarboxylase; AET, 2-aminoethylisothiouronium bromide.

toethanol was present. After electrophoresis, one gel strip (1 cm wide) was stained for protein with Coomassie blue and another gel strip was sliced into 1-cm squares and assayed for enzyme activity. The bands that contained enzyme activity were pooled together. Enzyme was extracted from the gel either by homogenizing the gel (about 200 μl/cm^2 of gel) in 0.1 M potassium phosphate buffer containing 1 mM 2-aminoethylisothiouronium bromide (AET) and 0.2 mM pyridoxal phosphate (pH 6.0) or by electrophoretic elution with agarose as supporting medium. Enzyme solutions thus obtained were concentrated and analyzed for protein pattern and enzyme activity on polyacrylamide gel as described below for analytical gel electrophoresis.

The conditions and the procedure for analytical gel electrophoresis were the same as those described above for the preparative gel electrophoresis except that the sample volume was reduced to 50 μl per well and the concentration of polyacrylamide was changed to 5%.

pH Optimum and Kinetic Studies. The pH optima of GADCase and CADCase were determined from a series of assays with pH varied between 5.5 and 8.5. A control experiment that contained all the components except enzyme was run at every pH point. For K_m and K_i determinations, the conditions were the same as those described under *Enzyme Assays* except that the concentrations of substrates varied.

Protein Determination. Protein was measured by using either a modification of the method of Lowry *et al.* (23) or, in the 0–5 μg range, the somewhat more sensitive Coomassie dye-binding technique of Bradford (24). Bovine serum albumin was used as the standard.

Preparation of Starting Material. The starting material was prepared according to the procedure described previously for GADCase preparation from mouse brain (25) with some additions and modifications. In a typical preparation, gray matter from bovine brain was dissected out and a 25% homogenate was made in ice-cold double-distilled water containing 0.02 mM pyridoxal phosphate, 0.1 mM AET, 1 mM reduced glutathione, and 0.1 mM EDTA (pH 7.2). All operations were carried out at 4°C, unless otherwise mentioned. The homogenate was centrifuged at 106,000 × g for 45 min and the supernatant fluid thus obtained was called the "crude extract." Concentrated potassium phosphate buffer at pH 7.2, AET, pyridoxal phosphate, reduced glutathione, and EDTA were added to the crude extract to give a final concentration as follows: 50 mM potassium phosphate, pH 7.2/1 mM AET/0.2 mM pyridoxal phosphate/1 mM reduced glutathione/1 mM EDTA (standard buffer). All the buffer solutions used in this study contained the same concentrations of AET, pyridoxal phosphate, reduced glutathione, and EDTA as those described above for the standard buffer, unless otherwise mentioned.

Ammonium Sulfate Fractionation. Solid ammonium sulfate was added gradually to the well-stirred crude extract solution to give ≈70% saturation. The pH of the solution was monitored continuously with a pH meter and was maintained at 7.2 by gradual addition of 0.1 M NH_4OH during the addition of ammonium sulfate as described (25). The precipitate was dissolved in and dialyzed against the standard buffer.

Production of Antibodies. Rabbits were injected biweekly with 3–50 μg of purified CADCase I/CSADCase or GADCase/CADCase II preparations in complete Freund's adjuvant into subscapular muscle as described (25). Animals were bled after the fifth injection. The production of antibodies was checked by immunodiffusion tests as described (25).

RESULTS

The successive steps in the purification of CADCase/CSADCase from bovine brain are summarized in Table 1. Ten steps were employed in the purification of CADCase/CSADCase and ≈0.36% of the total activity was recovered as a purified enzyme preparation representing 519-fold purification over the gray matter homogenate. For GADCase, a total of 12 steps was employed in the purification with 0.9% yield and 1,650-fold purification.

First Chromatography on Sephadex G-200. Sephadex G-200 gel was equilibrated with the standard buffer and packed into a 2.5 × 95 cm column. Approximately 30 ml (20 mg/ml) of concentrated crude extract was applied to the column. The column was eluted with the standard buffer and about 12 ml per fraction was collected. Both GADCase and CADCase activities appeared to be superimposed in both the low and high molecular peaks.

Second Chromatography on Sephadex G-200. Fractions containing GADCase and CADCase activities from the first Sephadex G-200 column were concentrated with ammonium sulfate (30–70%) and applied to a second Sephadex G-200 column (5.0 × 90 cm). Again, two peaks that contained both GADCase and CADCase activities were obtained as before. However, the position of the peak fractions in the low molecular region was separated by six fractions because the peak fraction for GADCase was at fraction 112 and that for CADCase was at 118 (Fig. 1*A*).

Chromatography on Hydroxyapatite. Hydroxyapatite was packed in a column (2.5 × 50 cm) with adapters to give a bed volume of 2.5 × 20 cm. The column was equilibrated with 10 mM phosphate buffer. The enzyme solutions from the major

Table 1. Purification of CADCase/CSADCase from bovine brain

Sample	Total activity,* units × 10^3	Total protein, mg	Specific activity,† (units/mg) × 10^3	Yield, %	Purification, fold
1. Gray matter homogenate	22,000	158,000	0.14	100	1
2. High speed supernatant	12,400	55,400	0.22	56	1.6
3. 1st $(NH_4)_2SO_4$ (0–70%)	11,500	45,000	0.26	52	1.9
4. 1st Sephadex G-200 (pool)	6,440	11,400	0.57	29	4.1
5. 2nd $(NH_4)_2SO_4$ (30–70%)	5,880	7,850	0.75	26	5.4
6. 2nd Sephadex G-200 (pool)	2,790	936	2.98	13	21
7. 3rd $(NH_4)_2SO_4$ (30–70%)	2,480	715	3.47	11	25
8. Hydroxyapatite (pool)	546	25	21.84	2.5	156
9. DEAE-cellulose (pool)	210	4.2	50.00	0.95	357
10. Preparative gel electrophoresis	80	1.1	72	0.36	519

* 1 unit = 1 μmol of product formed per min at 37°C.

† The specific activities of the peak fractions were: sample 4, 1.1; sample 6, 4.5; sample 8, 44; sample 9, 75.

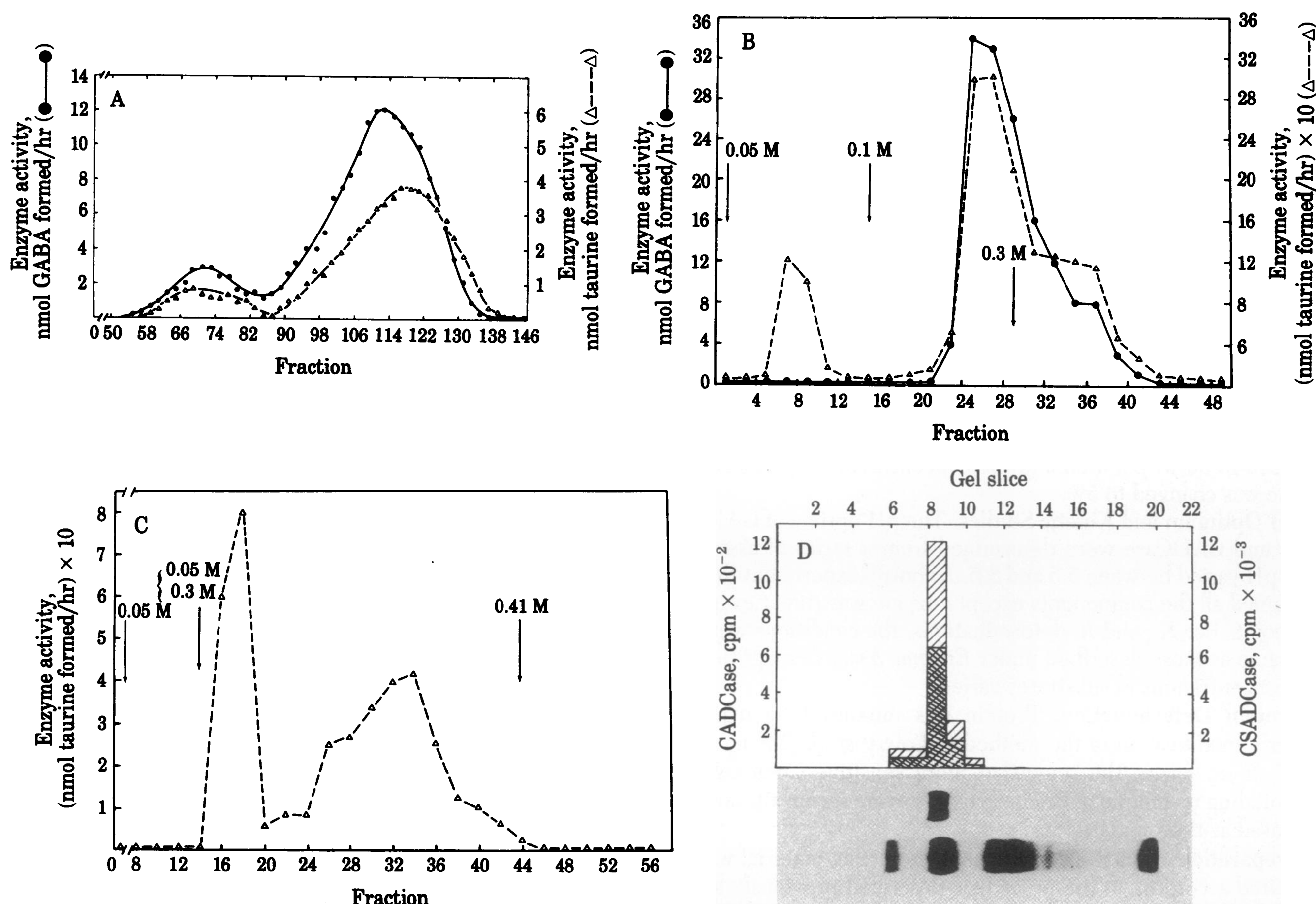

FIG. 1. (*A*) Second Sephadex G-200 column (5.0 × 90 cm) chromatography. The concentrated enzyme solution from the first Sephadex G-200 columns was applied and eluted with 0.05 M potassium phosphate buffer (pH 7.2) containing 1 mM AET, 0.2 mM pyridoxal phosphate, 1 mM reduced glutathione, and 1 mM EDTA (standard buffer). GADCase (●——●) and CADCase activities (△---△) were expressed as nmol of GABA formed per hr and nmol of taurine formed per hr, respectively. (*B*) Chromatography of GADCase/CADCase on hydroxyapatite gel. The concentrated enzyme solution from the second Sephadex G-200 column was dialyzed in 10 mM standard buffer and applied to the column. The column was first washed with 50 mM standard buffer (first arrow). Second arrow, start of elution with 100 mM standard buffer; third arrow, elution was continued with 300 mM standard buffer. GADCase (●——●) and CADCase activities (△---△) were expressed as nmol of GABA formed per hr and nmol of taurine formed per hr, respectively. (*C*) Chromatography of CADCase I on DEAE-cellulose. Fractions from the first peak (CADCase I, no GADCase activity) of the hydroxyapatite column were combined and applied to the DEAE-cellulose column (2.5 × 20 cm). The column was first washed with 50 mM phosphate standard buffer (first arrow). Second arrow, start of a linear gradient of standard potassium phosphate buffer from 50 to 300 mM; third arrow, elution was continued with 410 mM potassium phosphate buffer. CADCase activity (△---△) was expressed as nmol of taurine formed per hr. (*D*) Preparative gel electrophoresis. Five hundred microliters of the concentrated CADCase I solution from the first peak of the DEAE-cellulose column (*C*) was applied to a 7% polyacrylamide slab gel. The enzyme solution was in 10% glycerol containing bromphenol blue as marker. Before application of the sample, a current of 20 mA was passed through the gel for 30 min with buffer containing 25 mM Tris, 192 mM glycine, 0.065 mM reduced glutathione, 0.2 mM pyridoxal phosphate, and 0.1% 2-mercaptoethanol (pH 8.4). Electrophoresis was carried out at 4°C at 15 mA for the first hour and at 20 mA for an additional hour with the same buffer system as described except that no 2-mercaptoethanol was present. After electrophoresis, one gel strip (1 cm wide) was stained for protein with Coomassie blue (*Lower*) and another gel strip was sliced into 1-cm squares and assayed for CADCase (▨) and CSADCase activities (■). The bands that contained CADCase/CSADCase activities were pooled together. CADCase/CSADCase was extracted from the gel by homogenizing the gel (about 200 $\mu l/cm^2$ of gel) in 100 mM potassium phosphate buffer containing 1 mM AET, 0.2 mM pyridoxal phosphate (pH 6.0). The gel was removed by a brief centrifugation. CADCase/CSADCase solutions thus obtained were concentrated and analyzed for protein pattern (*Upper*) and enzyme activity on polyacrylamide gels as described above.

peak (the low molecular weight peak) of the second Sephadex G-200 column were concentrated as before and dialyzed in the equilibrating buffer before application to the hydroxyapatite column. The column was first eluted with 50 mM phosphate buffer (Fig. 1*B*, first arrow), followed by a linear gradient that was made of 100 ml each of 100 and 300 mM phosphate buffer (second arrow), and finally with 300 mM phosphate buffer (third arrow). There are two peaks containing CADCase activities. The first peak that is free of GADCase activity appeared after elution with 50 mM phosphate buffer (referred to as CADCase I). The second peak that appeared after elution with 100 mM phosphate buffer also contained GADCase activity. Furthermore, the GADCase and CADCase activities appeared to be superimposed in the second peak (referred to as CADCase II/GADCase) (Fig. 1*B*).

Chromatography of CADCase I on DEAE-Cellulose. DEAE-cellulose (DE-52) was equilibrated with the standard buffer and packed into a 2.5 × 20 cm column. Fractions 5–10 from the preceding step were combined and applied directly to the DEAE column. The column was first eluted with 50 mM phosphate buffer (first arrow), followed by a linear gradient that was made of 50 ml each of 50 and 300 mM phosphate buffer (second arrow). After the gradient, the column was further eluted with 410 mM phosphate buffer (third arrow). Two peaks containing both CADCase and CSADCase (not shown) activities were obtained (Fig. 1*C*).

Preparative Polyacrylamide Gel Electrophoresis of CADCase I/CSADCase. Fractions 28–36 from the preceding steps were combined and concentrated by ultrafiltration. The concentrated sample was applied to a 7% polyacrylamide slab gel as detailed in *Materials and Methods*. The enzyme solutions extracted from preparative gels either by electrophoretic elution or by homogenizing the enzyme-containing gel strip in the standard buffer were concentrated again with ultrafiltration. About 40% of CADCase/CSADCase activity was recovered (Fig. 1*D*).

Chromatography of GADCase/CADCase II on DEAE-Cellulose. The elution conditions were the same as those described for Fig. 1*C*. The size of the column used was 2.5 × 50 cm and the sample applied was the concentrated solution from fractions 25–33 of the hydroxyapatite column. The activities of GADCase and CADCase again comigrated throughout the entire elution spectra. Two activity peaks were obtained. The major peak centered around fraction 30 and a minor broad peak was around fraction 42.

Chromatography of GADCase/CADCase II on the Second Hydroxyapatite Column. The concentrated solution of the major peak (fractions 29–32) from the DEAE-cellulose column was applied to a second hydroxyapatite column. The conditions of the column were the same as those of the first hydroxyapatite column (see Fig. 1*B* for details). The protein solution was applied in 10 mM potassium phosphate buffer and washed with 30 ml of the same buffer. The column was further eluted with 90 and 120 ml of 50 and 100 mM potassium phosphate buffers, respectively. Finally, the column was eluted with 90 ml of a linear gradient from 100 to 300 mM phosphate buffer. Both GADCase and CADCase activities again comigrated with peaks around fractions 34 and 53.

CRITERIA OF PURITY

Polyacrylamide Gel Electrophoresis. About 50 μg of CADCase I/CSADCase solution obtained from preparative gel electrophoresis was applied to a 7% polyacrylamide slab gel. CADCase I/CSADCase migrated as a single protein band containing both the CADCase and CSADCase activities. No GADCase activity could be detected (Fig. 1*D Upper*). For GADCase/CADCase II, a 15-μg aliquot of the most purified GADCase/CADCase II solution (peak fraction 53 of the second hydroxyapatite column) was applied to a 5% polyacrylamide slab gel. A single protein band that contained GADCase, CADCase, and CSADCase activities was obtained as shown in Fig. 2. Furthermore, there were several fractions (fractions 52–55) with constant specific activity.

Determination of K_m Values. K_m values were determined from a Lineweaver–Burk double reciprocal plot (26). K_m values for CADCase I and II with cysteic acid as substrate were 0.22 and 5.4 mM, respectively. When cysteine sulfinic acid was used as substrate, the K_m values for CADCase I and II were obtained as 0.18 and 5.2 mM, respectively. The K_m for CADCase II with L-glutamate as substrate was 1.6 mM. L-Glutamate was a competitive inhibitor for CADCase II with a K_i of 0.45 mM when cysteic acid was used as substrate. CADCase I and II were found to be inhibited by cysteine sulfinic acid to an extent of 80% and 35%, respectively, at 0.45 mM when cysteic acid was used as substrate.

pH Profile. The pH optimum for CADCase I is around 7.4 with cysteic acid as substrate. For GADCase/CADCase II, relatively sharp pH profiles were obtained, with the optimum around 6.8 and 7.5 with L-glutamate and cysteic acid as substrates, respectively.

Immunodiffusion Test. Serum from rabbits that had been immunized with a total of 60 and 120 μg of purified CADCase

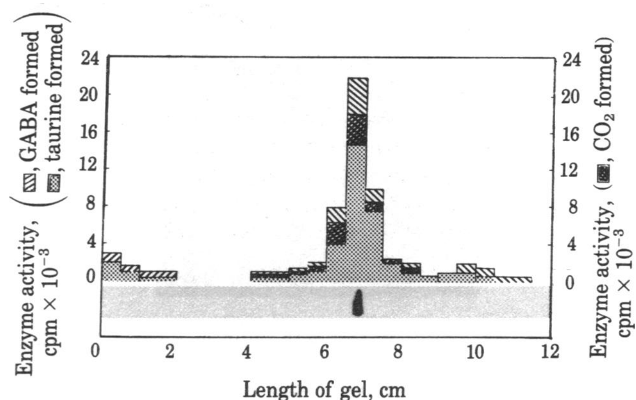

FIG. 2. Polyacrylamide gel electrophoresis of bovine GADCase/CADCase II. A 25-μg aliquot of the most purified GADCase/CADCase II solution (peak fraction of the second hydroxyapatite column) was applied to a 5% polyacrylamide slab gel. The protein pattern is at the bottom. Migration was from left (cathode) to right (anode). Enzyme activity was measured in slices of a parallel gel. GADCase (▨), CADCase (▩), and CSADCase (■) activities were expressed as cpm.

I/CSADCase appeared to contain antibody specific to CADCase I/CSADCase as shown in Fig. 3. No antibody could be detected with serum from rabbits that had been immunized with a total of 30 and 15 μg of CADCase I/CSADCase. Antibodies to GADCase/CADCase II could be detected in serum from rabbits that had been injected with a total of 50 and 100 μg of purified GADCase/CADCase II (Fig. 4). Furthermore, the precipitin line still retained the specific enzyme activity—e.g., CADCase or GADCase activity, suggesting that the immunocomplex was indeed a CADCase I/CSADCase anti-CADCase I/CSADCase or GADCase/CADCase II·anti-GADCase/CADCase II complex. The lack of immunoprecipitin line between CADCase I/CSADCase and anti-GADCase/CADCase II (well 6 in Fig. 3) or anti-CADCase I/CSADCase and GADCase/CADCase II (well 5 in Fig. 4) suggests a gross immunologic difference between CADCase I/CSADCase and GADCase/CADCase II.

Enzyme Inhibition Test. Enzyme inhibition testing was car-

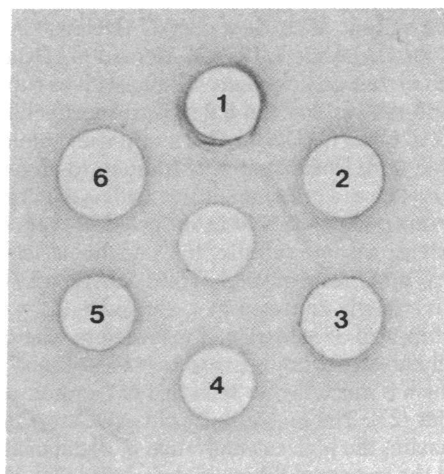

FIG. 3. Immunodiffusion test with antibody against CADCase I/CSADCase. The center well contained 30 μl of CADCase I/CSADCase solution from the hydroxyapatite column. The outer wells 1, 2, 3, and 4 contained 30 μl of serum from rabbits that had been immunized with a total of 60, 120, 30, and 15 μg of purified CADCase I/CSADCase, respectively. Wells 5 and 6 contained pre-immune serum and serum from rabbits that had been immunized with 100 μg of purified GADCase/CADCase II, respectively.

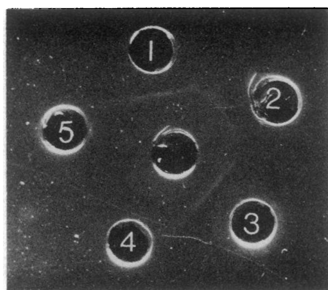

FIG. 4. Immunodiffusion test with antibody against GADCase/CADCase II. The center well contained 30 µl of GADCase solution from the hydroxyapatite column. Outer wells 1, 2, and 3 contained serum from rabbits that had been immunized with a total of 50 and 100 µg of purified bovine GADCase/CADCase II and 50 µg of purified mouse brain GADCase, respectively. Wells 4 and 5 contained pre-immune serum and serum from rabbits that had been immunized with 120 µg of purified CADCase I/CSADCase, respectively.

ried out as previously described (6). CADCase I/CSADCase activity was inhibited to a maximum of ≈60% by incubating with excess anti-CADCase I/CSADCase serum for 24 hr at 4°C. Almost all of the enzyme activity was precipitated, presumably in the form of CADCase I/CSADCase·anti-CADCase I/CSADCase complex. Anti-GADCase/CADCase II inhibited GADCase/CADCase II activity to an extent of ≈50% under the conditions as described (6). CADCase I/CSADCase activity was not inhibited by anti-GADCase/CADCase II. Similarly, anti-CADCase I/CSADCase did not have any effect on GADCase/CADCase II activity.

DISCUSSION

One of the important conclusions from the present study is that taurine and GABA are synthesized by two distinct enzyme entities—namely, CADCase I/CSADCase and GADCase/CADCase II, respectively. The following observations support the above conclusion that CADCase I/CSADCase is the enzyme responsible for the biosynthesis of taurine and GADCase/CADCase II is responsible for GABA biosynthesis. First of all, CADCase I/CSADCase has a much higher affinity for cysteic and cysteine sulfinic acids than does GADCase/CADCase II (K_m values for CADCase I/CSADCase and GADCase/CADCase II with cysteic and cysteine sulfinic acids as substrate are 0.22 and 0.18 mM vs. 5.4 and 5.2 mM, respectively). Second, although GADCase/CADCase II can also use cysteic and cysteine sulfinic acids as substrates in addition to glutamate, the affinity for cysteic and cysteine sulfinic acids is much lower than the affinity for L-glutamic acid as reflected in their K_m values (K_m for cysteic, cysteine sulfinic, and L-glutamic acids are 5.4, 5.2, and 1.6 mM, respectively). Third, GADCase/CADCase II activity is strongly inhibited by L-glutamate (K_i = 0.45 mM) with L-cysteic acid as substrate. Furthermore, the concentration of L-glutamate in brain tissue is ≈8–12 µmol/g of fresh weight, which is much higher than that of L-cysteic or cysteine sulfinic acids (27). The high affinity of GADCase/CADCase II for L-glutamate, the high concentration of L-glutamate in brain tissue, and the strong inhibition of decarboxylation of L-cysteic acid by L-glutamate argue strongly that GADCase/CADCase II is involved exclusively for GABA biosynthesis and not involved in the biosynthesis of taurine. On the other hand, CADCase I/CSADCase, which can use only L-cysteic and L-cysteine sulfinic acids but not L-glutamate as substrates, is the enzyme responsible for taurine biosynthesis.

The present study showing the lack of crossreactivity between anti-GADCase and CADCase I/CSADCase and vice versa will definitely help to clarify some doubts about the validity of immunocytochemical results which I, as well as others, have used extensively for the identification of GADCase-containing neurons and GABA-ergic pathways (for review, see refs. 7, 8, and 28). The availability of specific antibody to CADCase I/CSADCase will greatly facilitate the identification of the cellular and subcellular locations of the taurine-synthesizing enzyme, which may, in turn, shed light on the possible role of taurine as a neurotransmitter or modulator. Indeed, the taurine-synthesizing enzyme, CADCase I/CSADCase, in rat cerebellum has been localized at light and ultrastructural levels; the details of the immunocytochemical localization of CADCase I/CSADCase are described elsewhere (29).

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