Monoclonal antibodies to glutamic acid decarboxylase
(γ-aminobutyric acid/immunohistochemistry)

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ABSTRACT Five monoclonal antibodies that recognize chicken brain glutamic acid decarboxylase (GAD) have been selected and designated GAD-1 to -5. GAD-1 to -5 were selected on the basis of their ability to immunoprecipitate active GAD from crude brain extracts. GAD-1 recognizes an epitope that is conserved in many vertebrates; the epitope recognized by GAD-5 is restricted to the chicken. Radioimmunoassays with GAD-1 indicate that GAD is highly enriched in brain relative to other tissues. GAD was localized immunocytochemically with GAD-1 and GAD-2 in rat cerebellum, spinal cord, and retina. The staining pattern is in agreement with that obtained previously with polyclonal antisera to GAD. GAD from the chicken brain was purified by chromatography on an immunofinity column made of GAD-1. NaDodSO4/PAGE analysis of the immunofinity-purified GAD fractions shows a major band of 59 kDa and minor bands at 63 and 54 kDa.

The enzyme glutamic acid decarboxylase (GAD) catalyzes the conversion of glutamic acid to γ-aminobutyric acid (GABA) and CO2. The tissue and cellular distribution of GAD is highly restricted. GAD is localized largely to neurons and in particular to those that appear to use GABA as a neurotransmitter. Thus, the expression of GAD plays a key role in determining a neuron’s transmitter phenotype.

Many of the important kinetic and chemical properties of mammalian GAD were elucidated in the fundamental and pioneering studies of Roberts and his colleagues (reviewed in ref. 1). Subsequent work has raised the possibility that important kinetic variant forms of GAD exist (2–4). Purification of GAD by conventional biochemical methods is beset with a number of problems, including a relatively low starting concentration in the brain, a tendency of the enzyme to aggregate, and the necessity of multiple column separations to resolve GAD from other proteins. As a consequence, certain basic questions remain unanswered. Wu et al. (5) have purified mouse brain GAD to apparent homogeneity. However, NaDodSO4/PAGE analysis of the pure preparation reveals multiple bands ranging from 15 to 118 kDa (6). The relationship of these multiple bands to the active form(s) of the enzyme has not been elucidated. Antisera to this preparation have been extensively used to map the distribution of GAD in the brain (1). The exact range of polypeptides recognized by such antisera remains to be defined. No data on the amino acid sequence of GAD have been published.

Monoclonal antibodies (mAbs) to GAD could greatly facilitate research on many aspects of the enzyme and the neurons that contain it. In this report, we describe five mAbs (GAD-1 to -5) that recognize GAD. GAD-1 and -2 are useful for immunohistochemical localization of the enzyme. Immunofinity purification with GAD-1 has given new data on the molecular nature of GAD.

MATERIALS AND METHODS

Partial Purification of GAD from Chicken Brains. GAD was partially purified from chicken brains by methods adapted from those of Wu et al. (5) and Oertel et al. (7). Frozen adult chicken brains were obtained from Pel Freez (Rogers, AR). The frozen brains were suspended in distilled water/0.2 mM pyridoxal phosphate (PLP)/1 mM aminoethylisothiouronium bromide (AET) at 6.6 ml of water per g of brain and homogenized in a Brinkman Polytron at setting 8 for 30 sec. The homogenate was centrifuged at 130,000 × g for 60 min to sediment membranes, and the supernatant was saved for further purification. GAD activity was precipitated from the supernatant by the addition of ammonium sulfate (261 g/liter). The precipitated ammonium sulfate pellet was resuspended in standard buffer (SB; 0.05 M KPO4, pH 7.2/0.2 mM PLP/1 mM AET) and dialyzed against SB for 2 hr. The dialyzed ammonium sulfate fraction was loaded onto a column of DEAE Sephadex previously equilibrated with SB. The column was eluted with a 50–300 mM gradient of KPO4 buffer (pH 7.2) containing 0.2 mM PLP and 1 mM AET. Typically, GAD activity emerged as a sharp peak at ~100 mM KPO4. Ammonium sulfate (261 g/liter) was added to the DEAE peak fractions to precipitate GAD. The precipitated GAD was resuspended in standard buffer and loaded on an LKB Ultrogel AcA44 gel permeation column. This column was eluted with standard buffer. The specific activity of the Ultrogel peak fraction was 75 nmol [14C]GABA precipitated/mg GAD/hr.

Assay for GAD Activity. GAD activity was assayed by measurement of [14C]GABA released from labeled glutamate by established methods (8, 9). Enzyme reaction mixtures consisted of 1 ml of glutamic acid (20 mM; 0.1 μCi of L-[U-14C]glutamic acid; 1 Ci = 37 GBq) in SB/0.1 ml of enzyme solution. In some assays, glutamic acid labeled at the 1 position only (L-[1-14C]glutamic acid) was substituted for uniformly labeled glutamic acid. The molar amounts of product generated by using the two types of label were the same, indicating that all of the released CO2 derived from the carboxyl group attached to the α carbon of glutamic acid.

Immunization of Mice and Hybridoma Production. BALB/c mice were primed with Ultrogel peak fractions of GAD in complete Freund’s adjuvant injected into foot pads and i.p. Three months later, they were given booster injections containing 5.4 mg of partially purified GAD protein in incomplete Freund’s adjuvant. The spleen was taken 4 days later and fused with NS-1 myeloma cells by standard methods (10).

Screening Assay for Anti-GAD mAbs. Hybridoma supernatants were screened for their ability to immunoprecipitate GAD activity. One milliliter of each supernatant to be tested was added to 600 μl of a crude GAD prep (0.92 mg of protein; ~4.5 μg of GAD) and incubated for 16 hr at 4°C. Formalin-fixed Staphylococcus aureus (Cowan strain) was the kind gift of Proc. Natl. Acad. Sci. USA Vol. 83, pp. 8808–8812, November 1986 Neurobiology

Abbreviations: AET, aminoethylisothiouronium bromide; GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase; mAbs, monoclonal antibodies; PLP, pyridoxal phosphate.

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of Susan E. Cullen. Packed *S. aureus* (50 µl) and 450 µl of phosphate-buffered saline (PBS; 139 mM NaCl/2.7 mM KCl/8.1 mM Na₂HPO₄/1.5 mM KH₂PO₄) was added to each test tube and incubated with constant agitation for 2 hr at 6°C. Bacteria were pelleted by centrifugation at 1000 × g for 3 min and washed twice in 3 ml PBS to remove any enzyme not tightly bound to the cells. The washed pellet was resuspended in 1 ml of SB and injected into a GAD assay mixture containing L-glutamic acid (5 mM; 0.1 µCi of L-[1-¹⁴C]glutamic acid) in SB. The reaction was carried out for 1 hr at 37°C to measure GAD activity bound to bacteria.

**Antibody Binding Assays.** The indicated organs were homogenized in 10 ml of SB for each gram wet weight using the Brinkman Polytron at setting 8 for 30 sec. Homogenates were centrifuged at 9100 × g for 20 min to pellet membranes. Supernatants were quickly frozen for storage, with aliquots set aside for protein determination. To determine antigen in these samples, 100-µl samples of extract containing 150 µg of protein were incubated in the wells of Dynatech PVC multwell dishes for 60 min at room temperature. The wells were aspirated and washed twice with 200 µl of PBS/1% bovine serum albumin and left to incubate with this solution for 60 min at room temperature. Wells were washed again with PBS/1% bovine serum albumin and filled with 100 µl of PBS/1% bovine serum albumin. Indicated concentrations of labeled and unlabeled antibodies were introduced and incubated for an additional hour at ambient temperature. Wells were then washed four times with 200 µl of PBS/1% bovine serum albumin to remove unbound antibody, cut out of the dish, and assayed in a γ counter.

**Immunohistochemical Visualization of Antigens.** One-day-old chicks or adult rats were anesthetized and fixed by transcardiac perfusion of a fixative containing 1% paraformaldehyde, 0.01 M periodate, 0.075 lysine in 0.06 M NaPO₄ buffer (pH 7.4) (11). Antigen localization on 10-µm cryostat sections was determined by the methods described (12).

**Coupling mAbs to Agarose Beads.** GAD-1 ascites fluid (5 ml) containing ~125 mg of protein was diluted 1:1 with PBS. Saturated ammonium sulfate (10 ml) was added to precipitate IgG. After 1 hr at room temperature, the cloudy suspension was spun at 7000 × g for 20 min. The supernatant was discarded and the pellet was resuspended in 2.1 ml of distilled H₂O and dialyzed against PBS and then 0.1 M 3-(N-morpholino)propanesulfonic acid (Mops) at pH 7.5. The final dialysate contained 13.4 mg of protein per ml (28.1 mg total) and was substantially enriched in IgG. This solution was mixed with 5 ml of washed Affi-Gel 10 (Bio-Rad) for 1 hr at 10°C. The derivatized beads were spun out of solution and resuspended in 40 ml of 0.1 M Mops/0.1 M ethanamine, pH 7.5, and incubated for 1 hr at 10°C to block remaining active sites. The beads were finally washed and stored in PBS at 10°C; 4.7 mg of protein per ml was coupled to the beads.

**Immunoaffinity Purification of GAD.** Packed IgG derivatized Affi-Gel 10 beads (0.5 ml) were first washed to remove adherent noncovalently bound protein. The washing sequence consisted of two washes with 5 ml of SB, one wash with 5 ml of SB/1 M NaCl, two washes with 5 ml of SB, one wash with 5 ml of elution buffer (50 mM KPO₄/10 mM diethyliamine/0.2 mM PLP/1 mM AET/20 mM glutamic acid pH 7.1) and three additional washes with elution buffer. The washes were then mixed with 5 ml of the ammonium sulfate fraction of our standard GAD preparation for 30 min at 6°C. Beads were then washed to remove nonadherent proteins (two times with 5 ml of SB, one time with SB/1 M NaCl, two times with 5 ml of SB). The washed beads were packed into a column and eluted with three 1-ml aliquots (F1–F3) of elution buffer to remove bound GAD. All fractions were neutralized and analyzed for protein and enzyme content.

**Other Methods.** mAbs were purified on protein A sepharose columns (13). Purified mAbs were iodinated with ¹²⁵T by chloramine-T catalysis (14). NaDodSO₄/PAGE analysis was carried out according to ref. 15. Immunoblots were prepared as detailed in ref. 12.

**RESULTS**

**Selection of Hybridomas to GAD.** Hybridomas to GAD were produced by immunizing mice with partially purified preparations of chicken brain GAD and screening for antibodies that recognize GAD, as described in Materials and Methods. In one fusion, all 48 wells had hybridomas but only 10 gave a signal above background. All wells with a positive signal were cloned. Upon testing, clones from four separate wells were stable producers of anti-GAD activity. These were designated GAD-1 to -4, recloned, and established as frozen stocks. A fifth line, GAD-5, originated in a subsequent fusion using the same methods. Ascites fluids from each clone were produced and used in the experiments described below.

**Tissue Distribution of Antigen Determined by RIA.** GAD-1 was purified, iodinated, and used to determine the tissue distribution of its antigen in extracts of various organs from the chicken and rat. Table 1 shows that the antigen is greatly enriched in chicken brain relative to muscle, liver, gizzard, and small intestine. In the rat, the antigen is highly concentrated in brain relative to liver, muscle, small intestine, kidney, and spleen. GAD-1 recognizes an epitope that is highly conserved in the higher vertebrates, being present in chicken, rat, cat, rabbit, macaque, and mouse brains. It is either absent or present only in very low concentrations in the goldfish brain. In contrast, GAD-5 is highly specific to the chicken brain.

**Immunohistochemical Localization of GAD-1 and GAD-2 Binding in Chicken and Rat Brain.** GAD-1 and GAD-2 stain appropriately fixed neural tissue from the chicken and rat intensely. Fig. 1 presents data from the rat, since GAD localization has been most intensively studied in this species. Sections stained with GAD-1 and GAD-2 show the same pattern, with GAD-2 giving slightly more intense staining. The major finding of this analysis is that GAD-1 and GAD-2 staining is highly selective for those structures that previous work has shown are positive for GAD (16–17). Structures reported to be negative fail to stain with GAD-1 and GAD-2. In the cerebellum, somata of Purkinje cells are intensely stained and those of stellate cells are positive but more lightly stained (Fig. 1A). Purkinje cell bodies are surrounded by a dense plexus of fibers and boutons; Purkinje cell main dendrites are outlined with boutons (presumably from stellate cells). Structures consistent with being glomeruli in the granule cell layer are intensely stained. The deep cerebellar nuclei contain an intense positive plexus of axons. The same set of structures stain with polyclonal sera to GAD (16). In the spinal cord, staining is restricted to the grey matter with the exception of some fine strands that project to the immediately adjacent white matter (Fig. 1C); the bulk of the white matter is negative. Within the grey matter, staining is found at all dorsoventral levels. However, the most intense staining is restricted to the dorsal horn. Again, this pattern resembles that seen with polyclonal sera (17). Staining in the neural retina is confined to neuron somata in the inner third of the inner nuclear layer and to fibers in the inner plexiform layer (Fig. 1D). This accords with the proposal that GAD in the rat retina is confined to amacrine cells (17).

**Characterization of Chicken Brain GAD by Immunoaffinity Chromatography.** Chicken brain GAD can be highly enriched by immunoaffinity chromatography on a GAD-1 column. Table 2 analyzes the purification of GAD from the first ammonium sulfate fraction of our standard enrichment procedure. Purification is performed by using beads coupled to GAD-1. As a control, parallel manipulations are performed by using beads coupled to 29-13, an IgG myeloma protein that...
Table 1. Tissue and species specificity of GAD-1 and GAD-5

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Relative binding of GAD-1, %</th>
<th>Brain</th>
<th>Relative binding of GAD-1, %</th>
<th>GAD-5, %</th>
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<tbody>
<tr>
<td>Chicken</td>
<td>100</td>
<td>Chicken</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Brain</td>
<td>100</td>
<td>Rat</td>
<td>88.4</td>
<td>1</td>
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<tr>
<td>Liver</td>
<td>0.3</td>
<td>Cat</td>
<td>87.1</td>
<td>2.5</td>
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<tr>
<td>Muscle</td>
<td>0</td>
<td>Rabbit</td>
<td>82.7</td>
<td>2</td>
</tr>
<tr>
<td>Small intestine</td>
<td>3.4</td>
<td>Macaque</td>
<td>81.8</td>
<td>1.5</td>
</tr>
<tr>
<td>Gizzard</td>
<td>1.5</td>
<td>Mouse</td>
<td>71.6</td>
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<tr>
<td>Rat</td>
<td>100</td>
<td>Goldfish</td>
<td>0.6</td>
<td>—</td>
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</table>

Assays on indicated tissue extracts were performed as described in Materials and Methods. In the left half, each well received 0.018 µCi of 125I-labeled GAD-1 antibody (specific activity 2.2 µCi/µg). Half the wells for each sample also received 100 µl of 1:5000 unlabeled GAD-1 ascites fluid to block all specific binding. Specific binding is defined as counts bound with labeled antibody alone minus counts bound with labeled and unlabeled antibody. Specifically bound counts are expressed as percentages relative to the values for brain. In the right half, analogous assays were done with GAD-1 and GAD-5 on brains from different species. —, Not performed. Values are the averages of triplicate samples.

provides a "neutral" antibody to control for nonspecific adsorption. The ammonium sulfate fraction is incubated with GAD-1 or 29-13 beads. The beads are then washed to remove loosely adherent proteins and finally eluted at pH 11 to dissociate enzyme bound to the antibody. Approximately half of the enzyme activity is bound to the GAD-1 beads. The enzyme eluted from these beads (F1–F3) is enriched up to 194-fold. Recoveries are low (16.6% of total or 34.5% of enzyme bound to beads); this is likely to be an underestimate because simply raising GAD to high pH and reneutralizing it

Fig. 1. GAD-2 staining pattern in several regions of rat central nervous system. (A) Cerebellum. ml, Molecular layer; pcl, Purkinje cell layer; igl, internal granule layer; wm, white matter. (B) Cerebellum in which primary antibody is normal mouse serum. (C) Spinal cord. DH, dorsal horn; VH, ventral horn. (D) Neural retina. prl, photoreceptor layer; onl, outer nuclear layer; opl, outer plexiform layer; inl, inner nuclear layer; ipl, inner plexiform layer; gcl, ganglion cell layer. (Bars: A–C, 100 µm; D, 20 µm.)
Table 2. Immunoaffinity purification of GAD from ammonium sulfate fraction

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein concentration, mg/ml</th>
<th>Total protein, mg</th>
<th>Protein recovery, %</th>
<th>$^{14}$CO$_2$, µmol</th>
<th>Specific activity, nmol/min*mg$^{-1}$</th>
<th>Enrichment of enzyme activity</th>
<th>Recovery of enzyme activity, %</th>
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<tr>
<td>Input</td>
<td>11</td>
<td>55</td>
<td>100</td>
<td>22.3</td>
<td>13.5</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>GAD-1 flow-through</td>
<td>8.5</td>
<td>42.6</td>
<td>77.5</td>
<td>11.6</td>
<td>9.1</td>
<td>0.67</td>
<td>52</td>
</tr>
<tr>
<td>29-13 flow-through</td>
<td>9.2</td>
<td>46</td>
<td>84</td>
<td>18.9</td>
<td>13.7</td>
<td>1.01</td>
<td>85</td>
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<tr>
<td>GAD-1 F1</td>
<td>0.028</td>
<td>0.028</td>
<td>0.05</td>
<td>2.2</td>
<td>2619</td>
<td>194</td>
<td>9.9</td>
</tr>
<tr>
<td>GAD-1 F2</td>
<td>0.035</td>
<td>0.035</td>
<td>0.064</td>
<td>1.3</td>
<td>1238</td>
<td>91</td>
<td>5.8</td>
</tr>
<tr>
<td>GAD-1 F3</td>
<td>0.013</td>
<td>0.013</td>
<td>0.024</td>
<td>0.21</td>
<td>538</td>
<td>40</td>
<td>0.9</td>
</tr>
<tr>
<td>29-13 F1</td>
<td>0.013</td>
<td>0.013</td>
<td>0.024</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>29-13 F2</td>
<td>—</td>
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<tr>
<td>29-13 F3</td>
<td>—</td>
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The input is an ammonium sulfate fraction. Identical aliquots of this solution were adsorbed onto either GAD-1 or 29-13 derivatized beads for immunoaffinity purification as described in Materials and Methods. The total enzymatic activity (µmol of $^{14}$CO$_2$ released in 30 min) and specific activity of enzyme is given for each fraction. —, Values measured are below the limits of detection.

causes a loss of enzymatic activity. No enzymatic activity could be eluted from 29-13 beads, indicating that nonspecifically bound enzyme is absent from the final eluates.

Fractions from the purification were analyzed by NaDodSO$_4$/PAGE (Fig. 2). The input and flow-through fractions are complex mixtures of proteins. F1–F3 from the GAD-1 beads have a light band at 63 kDa, an intense band at 59 kDa, and several light diffuse bands centered at 54 kDa. F1–F3 from 29-13 beads have no visible bands. Eluates from GAD-1 beads not exposed to an enzyme fraction (lanes 7–9) have no bands, thus excluding the possibility that some of the bands in lanes 1–3 were due to components of the ascites fluid leaching off.

Identical experiments were performed using GAD-2 to -5 beads (data not shown). Because the conditions of elution have not been optimized, enrichments were not as high as for GAD-1 beads. However, in each case substantial enrichment of the same set of bands as in Fig. 2 as well as GAD activity was obtained. This evidence strengthens the association of the protein bands in lanes 1–3 with GAD.

The polyclonal antiserum to GAD designated 1440 (7) has been extensively used to map GABAergic neurons. Preparations enriched by the procedure of Table 2 were analyzed on immunoblots using antiserum 1440 (data not shown). The 1440 antibody stains the two protein bands at 63 and 59 kDa intensely. Preimmune serum does not stain. Therefore, the proteins recognized by GAD-1 are included in the set of proteins recognized by the 1440 antiserum.

**DISCUSSION**

This paper describes the selection and properties of mAbs that recognize GAD. Because GAD is extremely difficult to purify to homogeneity, we used partially purified enzyme to immunize mice for hybridoma production. Anti-GAD mAbs were then selected with a screening assay based on their ability to immunoprecipitate the active enzyme. We then investigated whether the selected mAbs had the properties expected based on the known distribution of GAD. RIAs show that GAD-1 recognizes an antigen that is highly enriched in the brains of chickens and rats relative to non-neural tissues. The existence of extraneuronal GAD has been claimed but is controversial (reviewed in refs. 6 and 18). The present results strongly support the position that non-neural tissues have at best low overall levels of GAD. It is still possible that elements of the peripheral nervous system express GAD at a level below the sensitivity of the RIA. Localization of the antigen recognized by GAD-1 and GAD-2 in the rat central nervous system was done by immunohistochemistry. Results show that the antigen is distributed in the cerebellum, spinal cord, and retina in a manner consistent with the known distribution of the enzyme. GAD-1 and GAD-2 will thus be useful reagents for analyzing the morphology of GAD containing neurons and synapses in the intact brain and in experimental situations such as tissue culture. GAD-1 and GAD-2 are directed to different sites on the enzyme as determined by RIA (D.I.G., unpublished results). Because two independent mAbs are directed to the same enzyme, co-localization with GAD-1 and GAD-2 provides a stringent criterion for the presence of GAD. This could be useful in cases of controversial localization of GAD (see ref. 19).

Work with polyclonal antisera suggests that some aspects of GAD structure are strongly conserved in vertebrate phylogeny since anti-rat and anti-mouse GAD antisera cross-react with GAD from other vertebrates. However, the distribution of single epitopes cannot be studied with polyclonal antisera. RIAs with GAD-1 show that at least one
epitope is conserved during the evolutionary divergence of a varied collection of higher vertebrates, including monkey, cat, rabbit, rat, mouse, and chicken. This conservation suggests an essential role for the epitope; significantly, binding GAD-1 to the chicken enzyme reduces catalytic activity by 50% (D.I.G., unpublished results). RIA results also show that at least one epitope, that recognized by GAD-5, has a phylogenetic distribution limited to the chicken. Data on the location and sequence of conserved and nonconserved regions will be useful in elucidating structureactivity relations in the GAD molecule.

Although attempts to purify GAD to homogeneity have been made, no consensus has emerged on the structure of the enzyme. GAD has been purified almost 1000-fold from mouse brain (5, 6). This preparation contains multiple bands on NaDodSO4/PAGE ranging from 15 to 118 kDa, leading to the interpretation that GAD is built up from 15-kDa subunits (6). There is a single report of purification from the human brain by conventional chromatography (20). The most highly purified preparation consists of a single band of 67 kDa on NaDodSO4/PAGE. The β-kinetic form of GAD from the hog brain contains a single 60-kDa band on NaDodSO4/PAGE (3). These differences could be due to true species variation, selective enrichment of different subforms during the multiprocess purification, or proteolytic degradation. Clarification of this issue remains an important goal.

In this study, GAD was purified on a GAD-1 immunofinity column. The protein that was specifically bound by this column was analyzed for overall protein concentration, enzymatic activity, and polypeptide composition by NaDodSO4/PAGE. Specific activity was enriched by at least 194-fold, but this is probably an underestimate of the enzyme enrichment because elution conditions reduce enzymatic activity. The specifically eluted proteins in F1, F2, and F3 contain a complex of bands: the most intense at 59 kDa, a light band at 63 kDa, and several light diffuse bands centered about 54 kDa. In a parallel experiment, the enzyme was also passed over control beads derivatized with a "neutral antibody." Gels of the eluted control column are devoid of bands, showing that the bands from the GAD-1 column were specifically bound by the antibody. This raises the important question "What is the relationship of these bands to each other?" GAD may exist as a number of structural and kinetic variants (2-4). One interesting possibility is that these bands represent different variants. It is also possible that some of the bands represent polypeptides noncovalently associated with the GAD catalytic subunit. Although purification was rapid and a protease inhibitor was present throughout, some bands might still be degradation products of GAD. The fact that rapid purification can be achieved with good yield should allow us to find the correct explanation for the multiplicity of bands.

We thank Mary Everly Bane and Steven Mick for excellent technical assistance. Mr. Jonathan Hughes contributed to the procedure for purifying GAD. Dr. Donald Schmechel provided the anti-GAD antiserum. This work was supported by Grant NS12867 from the National Institute of Neurological and Communicative Disorders and Stroke.