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Original Articles

Antisera to γ-Aminobutyric Acid.

I. Production and Characterization Using a New Model System[1]

ANTHONY J. HODGSON, BOTOND PENKE, ANNA ERDEI, IAN W. CHUBB, and PÉTER SOMOGYI

Centre for Neuroscience (A.J.H.; I.W.C.; P.S.), Unit of Human Physiology, School of Medicine, The Flinders University of South Australia, Bedford Park, South Australia, 5042, Department of Medical Chemistry (B.P.), Medical School of Szeged, Szeged, Hungary, Department of Immunology (A.E.), Lóránd, Eötvös University, Göd, Hungary, and 1st Department of Anatomy (P.S.), Semmelweis Medical School, Budapest, Hungary

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Antisera to the amino acid γ-aminobutyric acid (GABA) have been developed with the aim of immunohistochemical visualization of neurons that use it as a neurotransmitter. GABA bound to bovine serum albumin was the immunogen. The reactivities of the sera to GABA and a variety of structurally related compounds were tested by coupling these compounds to nitrocellulose paper activated with polylysine and glutaraldehyde and incubating the paper with the unlabeled antibody enzyme method, thus simulating immunohistochemistry of tissue sections. The antisera did not react with L-glutamate, L-aspartate, D-aspartate, glycine, taurine, L-glutamine, L-lysine, L-threonine, L-alanine, α-aminobutyrate, β-aminobutyrate, putrescine, or δ-aminolevulinate. There was cross-reaction with γ-amino-β-hydroxybutyrate, 1–10%, and the homologues of GABA: β-alanine, 1–10%, δ-aminovalerate, ~ 10%, and ε-amino-caproate, ~ 10%. The antisera reacted slightly with the dipeptide γ-aminobutyrylleucine, but not carnosine or homocarnosine. Immunostaining of GABA was completely abolished by adsorption of the sera to GABA coupled to polyacrylamide beads by glutaraldehyde. The immunohistochemical model is simple, amino acids and peptides are bound in the same way as in aldehyde-fixed tissue and, in contrast to radioimmunoassay, it uses an immunohistochemical detection system. This method has enabled us to define the high specificity of anti-GABA sera and to use them in some novel ways. The model should prove useful in assessing the specificity of other antisera.

KEY WORDS: GABA; Antisera; Immunocytochemical model; Specificity; Amino acids; Dipeptides; Immunohistochemistry; Glutaraldehyde fixation; Nitrocellulose.

Introduction

The amino acid gamma-aminobutyric acid (GABA) is a major neurotransmitter in the central nervous system where its inhibitory action is mediated through a GABA receptor associated with a chloride ionophore in the membrane of the postsynaptic cell (13). GABA-releasing and GABA-sensitive neurons have been found in all areas of the mammalian nervous system (6,13). A full understanding of these pathways requires the identification of GABAergic neurons and their place in the neuronal circuitry. Until recently there have been two major methods for the cytochemical identification of GABAergic neurons: one method is based on the capacity of GABAergic neurons in many areas to take up GABA by a high affinity system, which is exploited by applying exogenous [^{3}H]GABA, followed by autoradiographic localization of labeled GABA within the neurons (5,11). The other method is based on the immunocytochemical localization of L-1-glutamate decarboxylase (GAD; EC 4.1.1.15), the GABA synthesizing enzyme that is present in GABAergic neurons and their terminals in high concentrations (19,40,41). The latter method has received wider acceptance because it is based on localization of an endogenous molecule. There have been difficulties, however, in the application of the method and interpretation of the results of GAD immunocytochemistry. It should be noted, for example, that it is the enzyme rather than the neuroactive molecule itself that is localized and the ratio of their concentrations may differ in different cellular compartments. It is also possible that GAD activity and cysteine sulfinic acid decarboxylase II activity (EC 4.1.1.29) (the activity capable of synthesizing the putative neurotransmitters taurine and hypotaurine) may be properties of one common protein molecule

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((3,37) see also ref. 39). Another problem is that GAD is sensitive to fixation and processing conditions (19); for example, some anti-GAD sera reveal GAD-containing cell bodies only after axoplasmic transport is blocked by injecting the animal with the cytotoxin colchicine.

We have used both of the above techniques to identify different types of GABAergic neurons in complex structures (9,29). The limitations of these techniques compromise results when they are used in combination with other procedures; for example, we have been unable to demonstrate GAD immunoreactivity in tissue postfixed with osmium. We have worked on developing antisera to GABA as another method for the localization of GABAergic neurons in order to increase the versatility of immunocytochemical methods. The use of anti-GABA sera seemed a promising strategy for structures where only a combination of procedures could be used to identify the different types of GABAergic neurons (9). Recently, others have reported the direct immunohistochemical demonstration of GABA in tissue sections using antisera directed against GABA (26,33).

In the present article the development of antisera to GABA will be described together with its immunocytochemical characterization using a nitrocellulose model system to simulate tissue sections. In the following article (30) the localization of GABA as revealed by these sera will be compared with the localization of GAD in the cerebellar and cerebral cortex, two areas where GABAergic neurons and neurotransmission have been extensively studied previously. Finally, in the third article (28) we will describe two novel immunocytochemical applications of the anti-GABA sera in the cortex, a brain area where GABAergic circuits have probably reached their greatest complexity. A preliminary report of some of these results has been published (10).

Materials and Methods

Chemical compounds and immunohistochemical reagents. The suppliers of the reagents used in this study were as follows: Glutaraldehyde, EM grade, from TAAB (Reading, England); polyacrylamide beads Bio Gel P200, 100-200 mesh, and *N*-hydroxysuccinimide-activated agarose, Affigel 10, from BioRad Lab. (Richmond, CA); nitrocellulose paper, 0.22 μm pore, from Sartorius (Göttingen, F.R.G.); bovine serum albumin (BSA), Cat. No. A4378, poly-L-lysine hydrobromide (mol wt 150,000–300,000), L-lysine, β-alanine, ϵ-amino-*n*-caproic acid, δ-amino-*n*-valeric acid, D-aspartic acid, putrescine, L-α-amino-*n*-butyric acid and DL-β-amino-*n*-butyric acid from Sigma Chemical Co. (St. Louis, MO); L-glutamic acid, L-aspartic acid, L-glutamine, taurine, DL-γ-amino-β-hydroxybutyric acid, glycine, δ-aminolevulinic acid, L-threonine, L-alanine, L-carnosine, γ-aminobutyryl-L-histidine sulfate, and γ-aminobutyryl-L-leucine hydrobromide from United States Biochemical Corporation (Cleveland, OH); γ-amino-*n*-butyric acid and 3,3′-diaminobenzidine tetrahydrochloride (DAB) from Serva (Heidelberg, F.R.G.); [$^3H_{2,3}$]GABA, 3.7 TBq/mmol, from Amersham International (England); rabbit peroxidase–antiperoxidase (PAP) from Dakopatts (Denmark); immunoglobulin (IgG) fraction of sheep anti-rabbit IgG from Silenus (Dandenong, Australia); where possible all other reagents were of analytical grade.

Synthesis of immunizing conjugates. Amino acids were coupled to BSA by using glutaraldehyde: BSA (60 mg, 0.87 μmol), GABA (100 mg, 971 μmol containing 3.7 KBq [^{3}H]GABA) and glutaraldehyde (50 mg, 500 μmol) were dissolved in 4.0 ml of phosphate buffer, 0.15 M, pH 8.0, and incubated in the dark at room temperature for 20 hr. Glutamate was coupled to BSA in an identical way. After reaction the yellow colored conjugate was extensively dialyzed against phosphate buffered isotonic saline.

Immunization of animals. Rabbits were immunized by initially injecting them with 0.5 to 1 mg of conjugate, either GABA or glutamate, emulsified in complete Freund's adjuvant and subsequently injecting antigen in incomplete adjuvant into multiple intradermal sites every 2 weeks. After 5 months the animals were bled. Anti-glutamate sera were used in this study merely as a control for the specificity of the immunohistochemical model.

Testing for specificity. All antisera reacted with BSA in immunodiffusion and were tested in an enzyme-linked assay (ELISA) (8,32) with both BSA–GABA conjugates and thyroglobulin–GABA conjugates using peroxidase-conjugated antibodies. To assess the specificity of the sera using an ELISA would require the synthesis of as many different conjugates as compounds tested. We therefore designed a more straightforward test system whereby any desired compound could be spotted onto a strip of activated paper. This new test system involved the same reagents and same manipulations as applied to histological sections and thus provided a versatile model for immunochemical reactions. The procedure consisted of first activating strips of nitrocellulose paper with polylysine and glutaraldehyde, then spotting on test compounds, and finally incubating the strips with antisera on a schedule based on the unlabeled antibody enzyme technique (31).

Activation of paper. Nitrocellulose paper was activated by incubation in the following series of solutions: poly-L-lysine 100 μg/ml in water overnight at room temperature; water for three 10 min washes; glutaraldehyde 10% in sodium hydroxide/potassium chloride buffer 0.2 M, pH 10.0, for 60 min; the same buffer for a minimum of three 10 min washes and then water for a similar period. Test compounds were then spotted on to dry paper as described below. Excess aldehyde groups were blocked by incubating the paper in 1.0 M lysine in 0.1 M sodium bicarbonate buffer, pH 8.3–8.5; the paper was washed in phosphate buffered isotonic saline containing Tris (10 mM) and potassium chloride (5.4 mM) (TPBS), pH 7.2–7.4, six times for 5 min, and protein binding sites blocked by incubation in gelatine (3% w/v) before incubation in the primary antisera. All results reported here were obtained with freshly prepared paper. Papers activated with glutaraldehyde could be stored dry at room temperature for several days or at −20°C under nitrogen for several weeks and were still useful for testing immunochemical specificity. These storage parameters were chosen arbitrarily.

Immunohistochemical staining. Primary antisera were diluted in TPBS that also contained normal sheep serum (20% v/v) and thimerosal (0.05% w/v) as a preservative. Strips of paper to which test compounds (0.5 μl) had been applied were incubated in the following sequence of solutions: Primary antiserum diluted 1/1000 (unless stated otherwise) overnight at room temperature; TPBS for six 5 min washes; sheep anti-rabbit IgG 1/30 for 120 min; TPBS for six 5 min washes; PAP complex diluted 1/50 for 120 min; six 5 min washes in TPBS; DAB (0.05%) in Tris buffer (50 mM, pH 7.4) for 5 min prior to addition of hydrogen peroxide (final concentration of 0.01%).

Synthesis of solid phase adsorbents. Polyacrylamide beads were activated with glutaraldehyde using a modification of an established method (34). Beads were incubated overnight at 56°C with glutaraldehyde (10% w/v) in sodium phosphate buffer, 0.1 M, pH 6.9. Compounds (200 mM) were coupled to the gel at pH 7.7–7.9 overnight at room temperature. Excess aldehyde sites were blocked with etha-

nolamine, 1 M, in sodium phosphate buffer, 0.1 M, pH 8.3. Adsorptions were performed by incubating overnight at 4°C a slurry of beads with an equal volume of serum diluted (normally 1/1000 final) in 10% normal sheep serum.

GABA, glutamate, β-alanine, and GABOB were coupled to Affigel 10 by using a twofold excess of amino acid (30 μmol/ml of gel) dissolved in organic solvent as described by the manufacturer. Adsorptions of sera were performed exactly as described for polyacrylamide beads.

Absorptions of antisera with free GABA in solution (10^{-5} to 10^{-2}M adjusted to pH 7.4) were carried out by incubation overnight at 4°C with dilutions of antibody.

Biochemical analyses. Amino acid analysis was performed on a Beckman 121M amino-acid analyser. Amino and amide groups of compounds spotted onto the paper were detected by the chlorine method described by Offord (20). Protein was determined using a dye binding method with BSA as the standard (4).

Results

Description of Antisera

Conjugates were produced that contained 20 mol of GABA or 65 mol of glutamate per mol of BSA. The sera of all nine animals that were immunized with GABA conjugate reacted in ELISA with GABA conjugated to thyroglobulin as well as to BSA–GABA, the immunizing conjugate. This showed that at least some of the antibodies were directed against the hapten and not the carrier. The antisera also reacted with BSA in gel diffusion, indicating that some of the antibodies were recognizing the carrier protein. Each antiserum was coded with a number corresponding to the rabbit immunized; serum GABA-3 was not tested extensively.

The antisera were characterized using the model system based on activated nitrocellulose paper. When the paper had been coated with polylysine, activated with glutaraldehyde, and spotted with GABA, the immunohistochemical reaction produced a brown spot, corresponding to the point of application of the GABA, on a pale background (Figure 1A). If the activation step was omitted, no immunoreaction was observed (Figure 2A). If nonimmune rabbit serum was substituted for anti-GABA serum or the PAP complex omitted from the schedule, no spots of reaction end-product were produced.

When GABA had been diluted so that decreasing amounts of the amino acid were applied to the paper, the intensity of the brown spot decreased in proportion (Figure 1A). The limit of detectability of GABA was 50 nmol. Applying more than 50 μmol of GABA produced no further increase in intensity of staining but caused smearing of the staining beyond the spotted area.

The intensity of staining of all spots decreased if the antisera were diluted. A dilution of 1/1000 was found to give optimal staining. With serum GABA-7, the limit of detection at a dilution of 1/1000 was 50 nmol, and this dilution revealed differences between another serum, GABA-8, for which the detection limit at 1/1000 was 5 μmol. Dilution of the serum GABA-7 to 1/4000 produced weaker but clearly discernible staining (detection limit 500 nmol). To compare the specificity and titer of the other sera, a dilution of 1/1000 was chosen. The intensity of staining of GABA varied between the sera. GABA-9 and GABA-7 showed the strongest staining and GABA-1 and GABA-8 the weakest (Figure 1A). In general, the background increased in proportion to the specific staining.

Different compounds were spotted onto the paper at a high concentration (50 μmol), a low concentration (500 nmol), and at 5 μmol if strong cross-reactivity was detected. The intensity of staining of spots of the variously diluted compounds was used to rank the cross-reactivity of the compounds. For example, β-alanine at 50 μmol (Figure 1B) produced a spot lower in intensity than 5 μmol of GABA (Figure 1A) and 500 nmol of β-alanine (Figure 1B) produced a spot much lower in intensity than 500 nmol of GABA (Figure 1A). This was assessed as showing that the cross-reactivity of GABA-7 antibody with β-alanine was less than 10% of that with GABA. Similar comparisons for all compounds tested are compiled in Table 1, which shows how the compounds are structurally related to GABA. All antisera cross-reacted with β-alanine and with γ-amino-β-hydroxybutyrate (Figure 1B). The intensity of staining of both compounds correlated with the intensity of staining of GABA. Thus strongly reacting sera (GABA-7 and GABA-9) gave moderately intense spots with 5 μmol of GABA, 50 μmol of β-alanine, and 50 μmol of GABOB, whereas weak sera (GABA-1 and GABA-8) showed weak reaction with GABA and β-alanine. Some sera also reacted with the dipeptide γ-aminobutyryl-L-leucine (GABA-Leu, Figure 1C) and the strongest reacting sera (GABA-7 and GABA-9) reacted weakly with δ-aminolevulinic acid. Subsequent amino acid analysis showed that the GABA-Leu contained significant amounts of the free amino acids GABA and leucine (Figure 3). In general the antisera showed parallel reactivities with all the compounds tested with one exception, GABA-6 reacted weakly with carnosine but not homocarnosine. No other sera reacted with either compound. Since the paper was blocked with lysine and showed little or no staining outside the spots, lysine can be included as a compound that did not react with the antiserum.

On the basis of these results GABA-7 was chosen for more extensive analysis. GABA-9 was also studied but to a lesser degree, since it showed greater reactivity with β-alanine than did GABA-7 (Figure 1B).

Among the extra compounds tested with GABA-7, two showed strong cross-reactivity. These two, δ-aminovaleric acid (5-aminopentanoic acid), and ε-aminocaproic acid (6-aminohexanoic acid), are homologous with GABA (as is β-alanine) but have longer carbon skeletons (Table 1). Their reactivity at 50 μmol was almost as strong as that with GABA at 5 μmol (Figure 1E, Table 1).

Adsorption experiments

Adsorption experiments were performed to confirm the specificity of the sera in both the model system and in immunocytochemistry (28,30).

When GABA coupled to polyacrylamide beads by glutaraldehyde was used as the solid phase (Figure 2A, GABA-Ads ii) adsorption of antisera resulted in the abolition of staining. However, adsorption of the sera to a solid phase adsorbent made by coupling GABA to agarose beads activated with *n*-

The following correction is made to the Results and to the text on figs. 1 and 2 on pages 231-236. The amounts of compounds listed should be multiplied by 10^{-3}; i.e. μmol should be replaced by pmol.

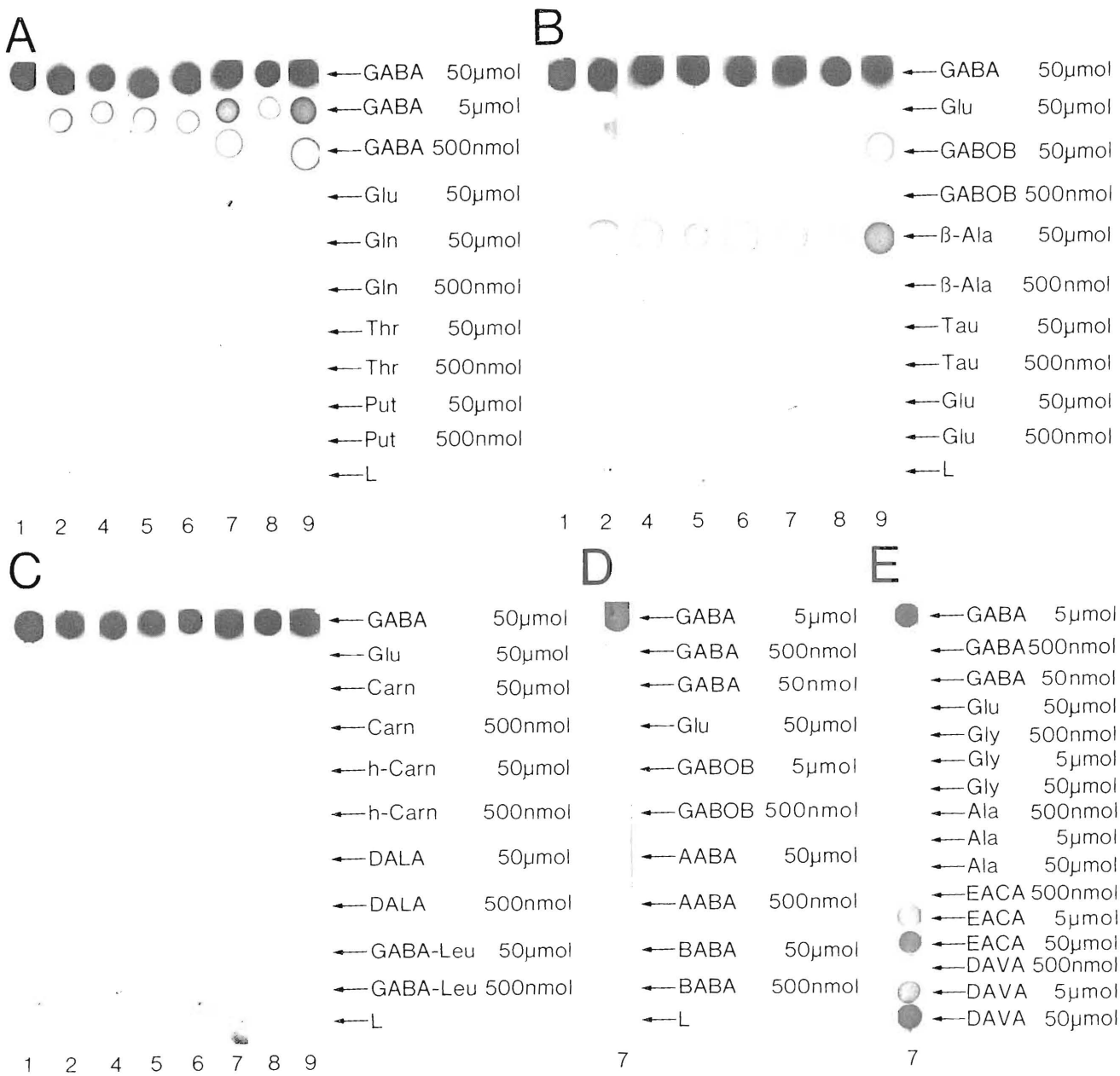
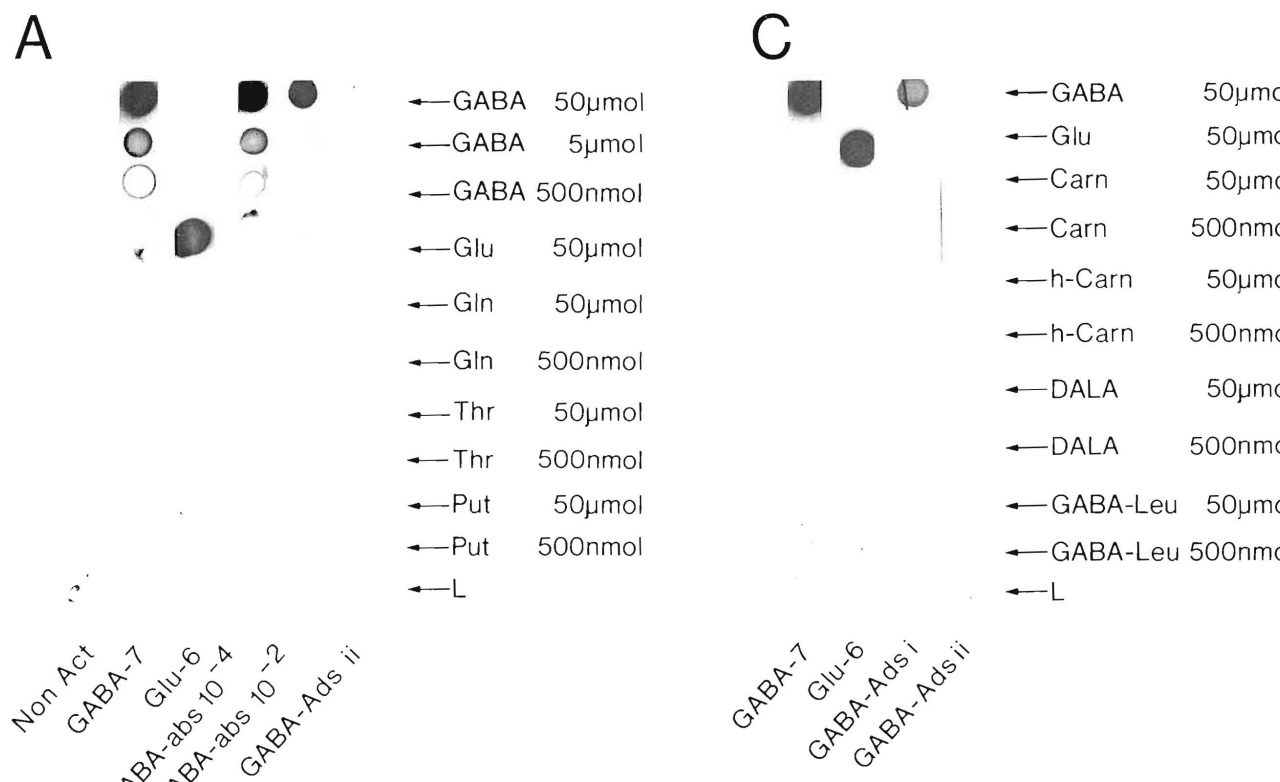


Figure 1. Reactivity of anti-GABA sera as detected by their immunoreactivity in the unlabeled antibody enzyme method (31). Strips of nitrocellulose, activated with polylysine and glutaraldehyde were spotted with 0.5 µl of solution containing the following compounds in the amounts shown: γ-aminobutyric acid, GABA; glutamate, Glu; glutamine, Gln; threonine, Thr; putrescine, Put; γ-amino-β-hydroxybutyric acid, GABOB; β-alanine, β-Ala; taurine, Tau; carnosine, Carn; homocarnosine, h-Carn; δ-aminolevulinic acid, DALA; γ-aminobutyryl-L-leucine, GABA-Leu; α-aminobutyric acid, AABA; β-aminobutyric acid, BABA; glycine, Gly; alanine, Ala; ϵ-aminocaproic acid, EACA; δ-aminovaleric acid; DAVA; L indicates the label used to identify the strips. The strips were incubated in eight different antisera raised in response to GABA conjugated to BSA. The numbers under each strip indicate which serum was used. **A**, **B**, and **C** were reacted with all sera. **D** and **E** show the reaction of serum GABA-7 only. Original magnification 1.4×.

hydroxysuccinimide ester (Affigel 10) only weakened the staining (Figure 2B, GABA-Ads i), despite the fact that incorporation of [^{3}H]GABA into the gel indicated that all of the reactive sites in the gel were saturated with GABA (22 µmol/ml).

Preincubation of the antiserum with BSA, glutamate, d-aspartate, taurine, and glycine coupled to polyacrylamide beads with glutaraldehyde had no effect on specific staining (Figure 2B). Adsorption with β-alanine or GABOB diminished staining of GABA and abolished staining of β-alanine (Figure 2B) or GABOB. However this effect was no different from the effect of diluting the serum or of partial adsorption to solid phase GABA (Figure 2B, GABA-Ads i, cf. β-Ala-Ads).



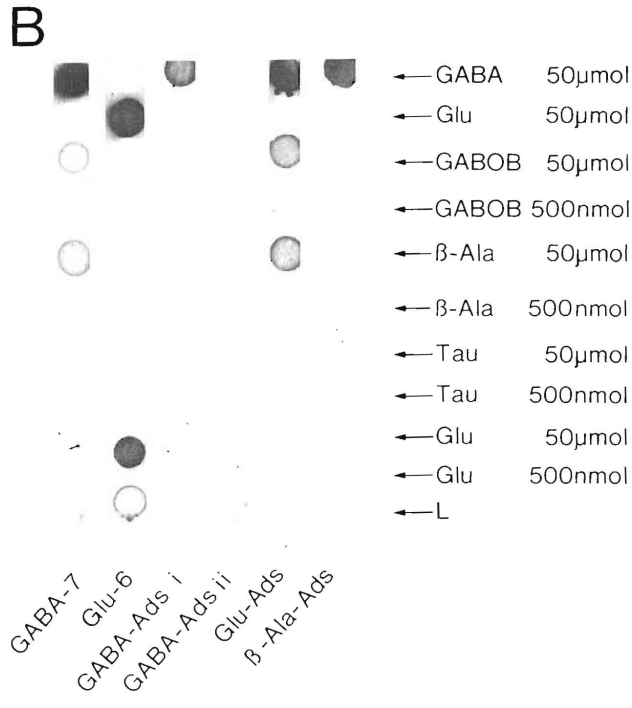


Figure 2. Effects of adsorption or absorption on the reactivity of anti-GABA sera. Strips were spotted with compounds and reacted exactly as described in Figure 1. **A** corresponds to compounds shown in Figure 1A, **B** to Figure 1B, and **C** to Figure 1C. The sera were treated as follows: Non Act: the nitrocellulose was not treated with polylysine or glutaraldehyde; GABA-7 positive control (cf. Figure 1); Glu-6: anti-GABA serum was replaced by an antiserum to glutamate; GABA-abs 10^{-4}: GABA-7 antiserum was pretreated with 10^{-4} M GABA in solution at pH 7.4 for 18 hr at 4°C; GABA-abs 10^{-2}: GABA-7 antiserum was pretreated with 10^{-2} M GABA. GABA-Ads ii: solid phase adsorption with polyacrylamide–glutaraldehyde–GABA beads. GABA-Ads i: solid phase adsorption with Affigel 10–GABA beads; Glu-Ads: solid phase adsorption with polyacrylamide–glutaraldehyde–glutamate; β-Ala-Ads: solid phase adsorption with polyacrylamide–glutaraldehyde–β-alanine beads.

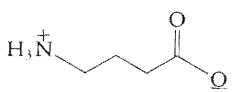
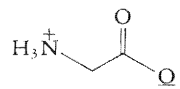
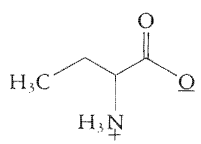
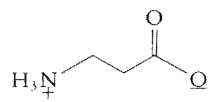
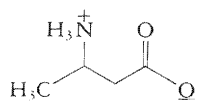
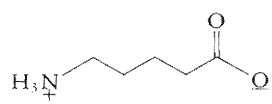
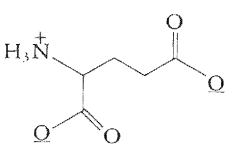
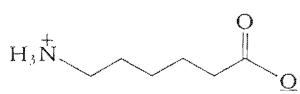
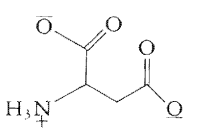
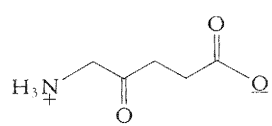
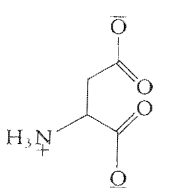
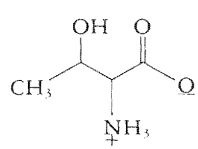
Absorption Experiments

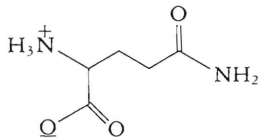
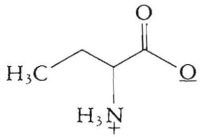
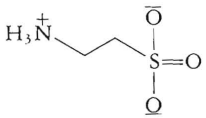
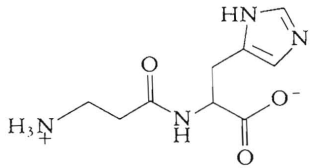
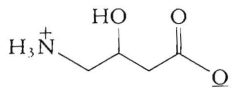
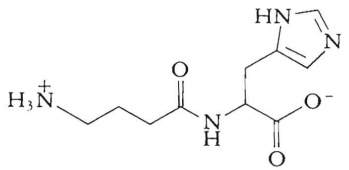
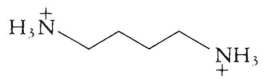
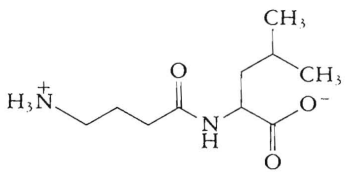
Preincubating GABA-7 antiserum with high concentrations of GABA (10^{-2} M) in solution at pH 7.4 diminished the intensity of staining of GABA in the model system but did not abolish it; thus a spot of 50 μmol of GABA still produced strong staining (Figure 2A). Preincubation with 10^{-4} M GABA had very little effect on staining (Figure 2A).

Further Tests of the Model System

To exclude the possibility that the lack of reactivity of the GABA antisera with compounds such as glutamate was caused by the failure of these compounds to bind to the paper, two additional controls were performed. First a method was used that detects N–C bonds in N-blocked amino acids and in peptides (20). Use of this method produced a pale blue spot at

Table 1. *Relationship between the sructure and reactivity of nitrocellulose-immobilized GABA derivatives with the GABA antibody*[a]

GABA derivative		Reactivity (%)	GABA derivative		Reactivity (%)
1. γ-aminobutyric acid		100	11. glycine		ND
2. L-α-aminobutyric acid		ND	12. β-alanine		1–10
3. DL-β-aminobutyric acid		ND	13. δ-aminovaleric acid		~ 10
4. L-glutamic acid		ND	14. ϵ-aminocaproic acid		~ 10
5. L-aspartic acid		ND	15. δ-aminolevulinic acid		1–10
6. D-aspartic acid		ND	16. L-threonine		ND

7. L-glutamine		ND	17. L-alanine		ND
8. taurine		ND	18. carnosine		ND
9. DL-γ-amino-β-hydroxybutyric acid		1–10	19. homocarnosine		ND
10. putrescine		ND	20. GABA-leucine		1–10

[a]The reactivity was evaluated by expressing the amount of a compound producing a spot of given intensity as a percentage of the amount of GABA from standard dilutions (Figure 1A) giving spots of an intensity that bracketed the intensity of the cross-reactive spot. Thus β-alanine, which at 50 μmol gave a spot of a density intermediate between that of 500 nmol (1%) and 5 μmol (10%) of GABA, was assessed to have between 1 and 10% reactivity with serum GABA-7. ND, not detectable.

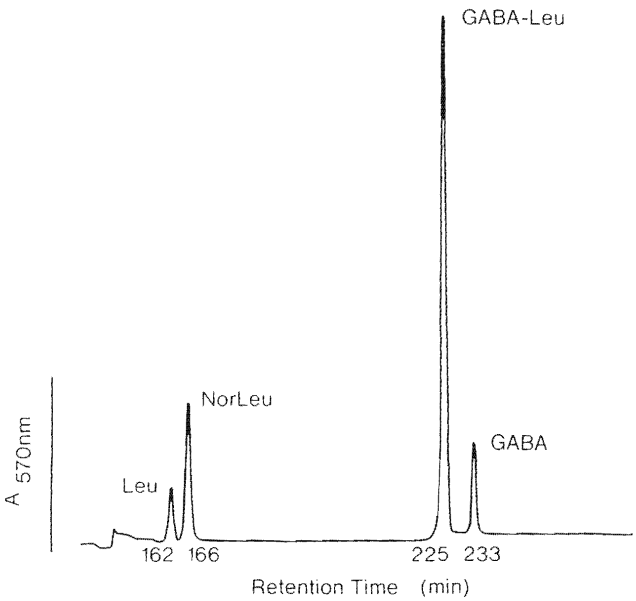


Figure 3. Amino acid analysis of γ-aminobutyryl-L-leucine hydrobromide (GABA-Leu). Norleucine (NorLeu) was included as the internal standard. Peak assignments are made on the basis of retention time in lithium buffers. Amino acids were detected by reaction with ninhydrin (absorbance max, 570 nm). Of the dipeptide material applied (10 mM), 0.64% was in the form of free leucine (Leu, retention time 162 min) and 1.3% in the form of free GABA (retention time 233 min), indicating that a significant amount of the material spotted onto the paper (Figures 1, 2) was in the form of free GABA.

the site of application of the compound. All spots of 50 μmol stained with a similar intensity except for putrescine, which has a free NH_2 and was much darker. Secondly, strips were incubated in an antiserum (Glu-6) that was produced in response to glutamic acid. This antiserum reacted very intensely with 50 μmol of glutamate but not at all with GABA (Figure 2A) nor any other compounds on the strips (Figure 2B,C), showing that the lack of reactivity of the GABA sera with glutamate was not due to failure of this compound to bind to the paper but was a genuine reflection of the specificity of the sera. Although similar antisera were not available for all compounds tested, indirect evidence for successful binding is provided by negative images of nonreactive spots on some strips that had high backgrounds (Figure 2B, Glu-Ads).

Although amino acids spotted on to strips of nonactivated nitrocellulose failed to produce immunoreactive spots, spots of BSA were reactive, confirming the results from immunodiffusion experiments which indicated that anti-BSA antibodies were present in the sera. One nanogram of BSA protein gave a clearly visible brown spot and 100 pg was discernible above background, indicating that the model system could be used to analyze specificity of anti-protein sera.

Amino Acid Analysis

The GABA used in specificity studies and in adsorptions was shown to be essentially pure by amino acid analysis, thus ensuring it did not contain any other amino acids as trace contaminants that may confuse interpretation. When β-alanine was similarly analyzed it was found to contain a trace of GABA. The percentage contamination in molar terms was only 0.05%, which is orders of magnitude less than the extent of reactivity on strips, 1–10% (Table 1). However the dipeptide GABA-Leu contained sufficient free GABA to account for the reactivity of this compound (Figure 3).

Discussion

The results can be summarized in two general points. First, the anti-GABA sera showed a very finely tuned specificity, reacting only with very closely related molecules, for example, GABOB, which differs from GABA by the inclusion of only one oxygen atom, or with β-alanine and δ-aminovaleric acid, which differ by only one or two methylene groups. Secondly, characterization of the sera was achieved by a method that uses the same reagents and a similar protocol to those used in immunocytochemistry, the intended final use of the sera, but which in contrast to tissue sections, utilizes a chemically defined system.

Specificity of the Anti-GABA Sera

The compounds tested for cross-reactivity with the sera were chosen as those that were both related in structure to GABA and naturally occurring and therefore potential reactants in animal tissues processed for immunocytochemistry. Drugs that are structurally related to GABA, although of potential interest, were not included. D-Aspartate was an exception in that we wished to study tissue from animals that had received injections of tritiated D-aspartate to show interrelationships between aspartate-accumulating neurons and GABA-containing neurons by combining GABA immunocytochemistry and autoradiography (12). The reactivity of the sera with the test compounds provides some information about the molecular nature of the antigenic determinant.

Reactivity with GABA

Although the antisera react with GABA conjugated with glutaraldehyde to proteins or to polylysine, there are indications that the antibody does not recognize the GABA molecule itself but rather the condensation product of GABA and glutaraldehyde. GABA, when free in solution, competes poorly with GABA fixed onto paper or into tissue sections (see ref. 30). High concentrations (10^{-2} M) were required before any effect on the intensity of staining was observed and at such high concentrations staining was only attenuated and not abolished. Hence there must be present in the sera high affinity antibodies that have low affinity for free GABA. Solid phase adsorption experiments confirmed this hypothesis. Adsorption of the antisera to GABA coupled to glutaraldehyde activated beads completely abolished staining, whereas similar beads coupled with glutamate had no effect. It is noteworthy that GABA coupled to Affigel 10 via an amide bond was not very efficient as an adsorbent despite high loading with GABA.

This indicates that the antibody recognizes, in part, the linkage of the GABA to glutaraldehyde. This is possible, since the antibody was generated in response to GABA that was conjugated to the carrier using glutaraldehyde.

It is not entirely clear what structure is formed between glutaraldehyde and amino groups. Since the bonds formed are stable in acid, Schiff bases cannot be involved and a structure formed by a Michael addition has been proposed (24). Alternatively the structure could be a Schiff base in conjugation with a double bond that is derived from the α,β unsaturated aldehyde polymer, the major reactive species in alkaline solutions of glutaraldehyde (18); this resonance structure is acid stable. Whichever bond is formed between GABA and glutaraldehyde, if our antisera recognize part of the structure, then they may be useful in investigating the nature of the structure.

Since our antisera react with the homologues of GABA, β-alanine, δ-aminovalerate, and ϵ-aminocaproate, it is possible that immunostaining of tissue sections could be wrongly attributed to GABA when in fact one of the cross-reactive moities was being localized. There are two possibilities: i) a small fraction (ca. 10%) of the antibodies in the polyclonal sera react with β-alanine as well as GABA but the remaining antibodies react monospecifically with GABA, or ii) the majority of antibodies react with β-alanine but with a lower affinity than for GABA. To distinguish between them we performed solid phase adsorptions to cross-reactive compounds. In all cases, adsorptions reduced in parallel reactivity to all reactive species. Thus adsorptions to β-alanine abolished all reactivity to β-alanine and GABOB but also substantially reduced GABA staining. This indicates that the majority of antibodies have some weak affinity for β-alanine. Since all sera tested showed similar reactivities with β-alanine, the production of antibodies that do not recognize the homologues of GABA may require monoclonal techniques. None of the other sera produced in response to GABA conjugates (26,33) are monoclonal, yet none have been tested, so far, with β-alanine or any other homologue save glycine. Our experience with eight rabbit sera indicate that this test is of crucial importance.

Compounds Reacting with the Antisera

δ-Aminovaleric acid (13 in Table 1) and ϵ-aminocaproic acid (14 in Table 1) reacted with the antisera but not as strongly as did GABA. These compounds are one and two methylene groups longer than GABA. β-Alanine (12 in Table 1), one methylene group shorter than GABA, was also reactive but less than δ-aminovaleric acid. Glycine (11 in Table 1), two methylene groups shorter than GABA, did not react.

Thus, it can be seen that as the length of the methylene chain increases from glycine to GABA, the compound becomes more reactive with the antisera. Compounds longer (13 and 14 in Table 1) than GABA are still reactive, but inclusion of a carbonyl group in δ-aminolevulinic acid (15 in Table 1) diminishes the reactivity. Addition of a hydroxyl group to GABA in GABOB considerably reduces the reactivity of the sera. Considering that this entails the addition of only one oxygen atom, it indicates the very finely tuned specificity of the antibody.

Compounds not Reacting with the Antisera

This fine specificity is also illustrated by those compounds that showed no reactivity with the antisera. α-Aminobutyric acid and β-aminobutyric acid (2 and 3 in Table 1) did not react, despite being isomers of GABA with the same molecular composition. Glutamate differs from GABA only by the addition of a carboxylate group to the γ position of GABA, the addition of this group, which carries a negative charge at physiological pH, abolishes all immunoreactivity. Likewise, addition of a carboxylate anion to the β position of β-alanine to form aspartate (5 and 6 in Table 1) also abolishes reactivity. Substitution of the carboxylate anion by sulfonate in β-alanine to form taurine also abolishes reactivity. The carboxylate anion appears essential, however, since its substitution by an amino moiety in putrescine (10 in Table 1) and a potential precursor of GABA (27) abolishes immunoreactivity. Glutamine, a potential source of glutamate and thence GABA, did not react with the sera. The L-amino acids alanine and threonine, found ubiquitously, and structurally similar to β-alanine and GABOB, did not cross-react even though β-alanine and GABOB did react.

The dipeptide homocarnosine (19 in Table 1) did not react even though it contains the GABA structure, nor did carnosine (18 in Table 1), which contains β-alanine. The dipeptide GABA-Leu probably does not react. The reactivity observed when samples of this compound were spotted onto paper was probably caused by free GABA that contaminated the sample (Figure 3).

Characteristics of the Test System

Although a critical assessment of the specificity of antibody binding is a cornerstone of the immunocytochemical method there have been few methods that fulfill all requirements. Radioimmunoassay (RIA) is widely used, especially for small peptides and amines, yet the technique has major drawbacks (22,23,25). Recently two procedures have been proposed as systems that avoided some of these drawbacks. Both methods incorporate aldehyde fixation.

The first system (15) involves spotting compounds onto filter paper and has obvious similarities to the present method. Differences are that the vapor phase was used to deliver the fixative and that ordinary filter paper was used. Our system uses nitrocellulose and fixation with solutions of glutaraldehyde. Glutaraldehyde cannot exist as a polymer in the vapor phase, yet it is polymeric glutaraldehyde that is believed to be the critical agent in alkaline solutions of the fixative (18).

The second system (25) is based on a protocol whereby test compounds are incorporated into gelatin, fixed with formaldehyde, and stained by immunofluorescence. This system could be quantified by spectrofluorometry. It remains to be shown whether this system can be used with peroxidase detection methods and glutaraldehyde fixation. It is noteworthy

that the above method was used to show that antibodies to 5-hydroxytryptamine (5HT) recognize the condensation product of this compound with the fixative formaldehyde with higher affinity than the free amine. Similar conclusions were reached (17) using agglutination of antigen-coated erythrocytes to test the specificity of anti-5HT antibodies.

From the findings reported here on GABA together with a brief report by others (26), it would seem that most antisera to small molecules recognize part of the chemical bridge used to conjugate the compound to the carrier protein. It would thus seem logical to discontinue the use of methods of cross-linking, such as by cyanogen bromide or carbodiimides, that introduce linkages which would not be found in tissue fixed by aldehydes if it is intended to use the antibody for immunohistochemistry.

Implications for Immunohistochemistry on Tissues

None of the putative neurotransmitters, glutamic acid, aspartic acid, taurine, carnosine, and glycine reacted with any of the antisera. These compounds are found in high concentrations in regions of the nervous system and some may act as the neurotransmitters in types of neurons that subserve a similar function to GABAergic neurons (2,6).

Of the homologues of GABA, β-alanine is the most likely candidate for a neurotransmitter in its own right, although in comparison with GABA, the evidence is weaker (2,6,7). The content of β-alanine in brain tissue, although lower than that of GABA (β-alanine/GABA = 0.03–0.08; ca. 0.04–0.1 μmol/g wet weight versus 1–2.5 μmol/g wet weight) (16,21), is comparable with that of other neurotransmitters such as acetylcholine and noradrenaline, and β-alanine appears to fulfill many of the criteria for a neurotransmitter (7). However it is not known how β-alanine is synthesized, so whether β-alanine is present in the same cells as GABA or in different ones is unknown. Because the concentration of β-alanine is 20- to 30-fold lower than GABA and the sera 10-fold less reactive with β-alanine it is possible that less than 1% of the staining in sections may be β-alanine.

Data concerning the presence in nervous tissue of the other homologues of GABA are scanty. Yoshino et al. (42) found no trace of GABOB (detection limit 0.01 μmol/g) in rat brain, but were able to detect β-alanine. δ-Aminovalerate and ε-aminocaproate were included in the present study because of their structural similarity to GABA. The latter has been used therapeutically as a fibrinolytic agent. Both compounds are synthesized and metabolized by bacteria (35,36). Their occurrence in mammalian tissues has been studied only sporadically (1,38) and it is not known whether they are found in the brain where they seem to be without major pharmacological effect (14).

In conclusion, we have produced antisera that recognize GABA fixed with glutaraldehyde in a model system. The antisera have a very fine specificity, recognizing only compounds that are very closely related to GABA. Of these cross-reactive molecules, only β-alanine poses a problem when the sera are applied on to tissues and this is a minor drawback. The model system is simple to use, can be quantified, and can be applied to other compounds such as peptide neurotransmitters (Hodgson, unpublished).

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Literature Cited

1. Ambert JP, Pechery C, Charpentier C, Hartmann L: Estimation of urinary amino acids. I. In the normal subject (technique of electrophoresis at pH 5.4, two-dimensional chromatography on silica gel thin layers, and in electrochromatography). Ann Biol Clin (Paris) 24:17, 1966
2. Bachelard HS: Biochemistry of centrally active amino acids. In Advances in Biochemical Psychopharmacology, Vol 29, Amino Acid Neurotransmitters. Edited by FV DeFeudis, P Mandel. Raven Press, New York, 1981, p 475–497
3. Blindermann JM, Maitre M, Ossola L, Mandel P: Purification and some properties of L-glutamate decarboxylase from human brain. Eur J Biochem 86:143, 1978
4. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:48, 1976
5. Cuénod M, Bagnoli P, Beaudet A, Rustioni A, Wiklund L, Streit P: Transmitter-specific retrograde labeling of neurons. In: Cytochemical Methods in Neuroanatomy. Edited by V Chan-Palay, SL Palay. Alan R Liss Inc, New York, 1982 p 17–44
6. Curtis DR, Johnston GAR: Amino acid transmitters in the mammalian central nervous system. Ergeb Physiol 69:97, 1974
7. DeFeudis FV, Martin del Rio R: Is β-alanine an inhibitory neurotransmitter? Gen Pharmacol 8:177, 1977
8. Engvall E, Perlmann P: Enzyme linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry 8:871, 1971
9. Freund TF, Martin KAC, Smith AD, Somogyi P: Glutamate decarboxylase-immunoreactive terminals of Golgi-impregnated axo-axonic cells and of presumed basket cells in synaptic contact with pyramidal neurons of the cat's visual cortex. J Comp Neurol 221:263, 1983
10. Hodgson AJ, Erdei A, Penke B, Chubb IW, Somogyi P: Antiserum to GABA: characterisation and immunocytochemical use on cat, monkey and human brain to identify GABA-ergic neurons. Soc Neurosci Abstr, in press
11. Iversen LL: Identification of transmitter-specific neurons in CNS by autoradiographic techniques. In Chemical Pathways in the Brain (Handbook of Psychopharmacology, vol 9). Edited by LL Iversen, SD Iversen, SH Snyder. Plenum Press, New York. 1978, p. 41–68
12. Kisvŕday ZF, Cowey A, Smith AD, Hodgson AJ, Somogyi P: Relationship between GABA immunoreactive neurons and neurons accumulating [^{3}H]-GABA or [^{3}H]-d-aspartate in striate cortex of monkey. Proc Cajal Conf Neurobiol, in press
13. Krnjević K: Chemical nature of synaptic transmission in vertebrates. Physiol Rev 54:418, 1974
14. Krnjević K, Phillis JW: Iontophoretic studies of neurons in the mammalian cerebral cortex. J Physiol 165:274, 1963

15. Larsson L-I: A novel immunocytochemical model system for specificity and sensitivity screening of antisera against multiple antigens. J Histochem Cytochem 29:409, 1981

16. Martin del Rio R, Orensanz Munoz LM, DeFeudis FV: Contents of β-alanine and γ-aminobutyric acid in regions of rat CNS. Exp Brain Res 28:225, 1977

17. Milstein C, Wright B, Cuello AC: Discrepancy between the cross-reactivity of a monoclonal antibody to serotonin and its immunohistochemical specificity. Mol Immunol 20:113, 1983

18. Monsan P, Puzo G, Mazarquil H: Etude du mécanisme d'établissement des liaisons glutaraldéhyde-protéines. Biochimie 57:1281, 1975

19. Oertel WH, Mugnaini E, Schmechel DE, Tappaz ML, Kopin IJ: The immunocytochemical demonstration of gamma-aminobutyric acid-ergic neurons-methods and application. In Cytochemical Methods in Neuroanatomy. Edited by V Chan-Palay, SL Palay. Alan R Liss Inc, New York, 1982, p 297–329

20. Offord RE: Amino acids, peptides and proteins. In Data for Biochemical Research, 2nd ed. Edited by RMC Dawson, DC Elliot, WH Elliot, KM Jones. Oxford University Press, Oxford, 1969, p 528

21. Perry TL, Hansen S, Berry K, Mok C, Lesk D: Free amino acids and related compounds in biopsies of human brain. J Neurochem 18:521, 1971

22. Petrusz P, Ordronneau P, Finley JCW: Criteria of reliability for light microscopic immunocytochemical staining. Histochem J 12:333, 1980

23. Pool CHRW, Buijs RM, Swaab DF, Boer GJ, Van Leeuwen FW: On the way to a specific immunocytochemical localization. In Immunohistochemistry. Edited by AC Cuello. Wiley, Chichester, 1983, p 1–46

24. Richards FM, Knowles JR: Glutaraldehyde as a protein cross-linking reagent. J Mol Biol 37:231, 1968

25. Schipper J, Tilders FJH: A new technique for studying specificity of immunocytochemical procedures: specificity of serotonin immunostaining. J Histochem Cytochem 31:12, 1983

26. Seguela PH, Geffard M, Buijs RM, Le Moal M: Antibodies against small molecules: an application to GABA. Soc Neurosci Abstr 9:404, 1983

27. Seiler N, Schmidt-Glenewinkel T, Sarhan S: On the formation of γ-aminobutyric acid from putrescine in brain. J Biochem (Tokyo) 86:277, 1979

28. Somogyi P, Hodgson AJ: Antisera to γ-aminobutyric acid: III. Demonstration of GABA in Golgi-impregnated neurons and in conventional electron microscopic sections of cat striate cortex. J Histochem Cytochem 33:249, 1985

29. Somogyi P, Freund TF, Kisvárday ZF: Different types of ^{3}H-GABA accumulating neurons in the visual cortex of the rat. Characterization by combined autoradiography and Golgi impregnation. Exp Brain Res 54:45, 1984

30. Somogyi P, Hodgson AJ, Chubb IW, Penke B, Erdei A: Antisera to γ-aminobutyric acid: II. Immunocytochemical application to the central nervous system. J Histochem Cytochem 33:240, 1985

31. Sternberger LA, Hardy PH Jr, Cuculis JJ, Meyer HG: The unlabeled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase) and its use in identification of spirochetes. J Histochem Cytochem 18:315, 1970

32. Stocker JW, Malanasi F, Trucco M: Enzyme immunoassay for detection of hybridoma products. In Immunological Methods, vol II. Edited by I Lefkovits, B Pernis. Academic Press, New York, 1981, p 299–308

33. Storm-Mathisen J, Leknes AK, Bore AT, Vaaland JL, Edminson P, Haug F-MS, Ottersen OP: First visualization of glutamate and GABA in neurones by immunocytochemistry. Nature 301:517, 1983

34. Ternyck T, Avrameas S: Polyacrylamide-protein immunoadsorbents prepared with glutaraldehyde. FEBS Lett 23:24, 1972

35. Tosa T, Chibata I: Utilization of cyclic amides and formation of ω-amino acids by microorganisms. J Bacteriol 89:919, 1965

36. Turner JM: Phenylglyoxal aminotransferase activity in Rhodopseudomonas spheroides Biochim Biophys Acta 77:697, 1963

37. Urban PF, Reichert P, Mandel P: Taurine metabolism and function. In Advances in Biochemical Psychopharmacology, Vol 29, Amino Acid Neurotransmitters. Edited by FV DeFeudis, P Mandel. Raven Press, New York, 1981, p. 537–544

38. Van Rheenan DL: High-voltage paper electrophoretic and paper chromatographic identification of biogenic amines in feces of normal dairy cattle. Rec Trav Chim 82:225, 1963

39. Wu J-Y: Purification and characterisation of cysteic acid and cysteine sulfinic acid decarboxylase and L-glutamate decarboxylase from bovine brain. Proc Natl Acad Sci USA 79:4270, 1982

40. Wu J-Y, Matsuda T, Roberts E: Purification and characterization of glutamate decarboxylase from mouse brain. J Biol Chem 248:3029, 1973

41. Wu J-Y, Lin CT, Brandon C, Chan TS, Mohler H, Richards JG: Regulation and immunocytochemical characterisation of glutamic acid decarboxylase. In Cytochemical Methods in Neuroanatomy. Edited by V Chan-Palay, SL Palay. Alan R Liss Inc, New York, 1982, p 279–296

42. Yoshino Y, DeFeudis FV, Elliot KAC: Omega-amino acids in rat brain. Can J Biochem 48:149, 1970