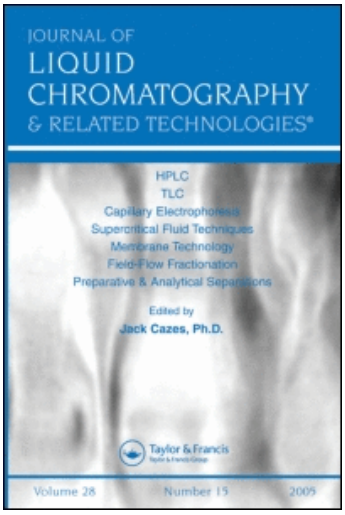

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A Simple Microassay for Glutamic Acid Decarboxylase by Ion Exchange Thin-Layer Chromatography

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A SIMPLE MICROASSAY FOR GLUTAMIC ACID DECARBOXYLASE BY ION EXCHANGE THIN-LAYER CHROMATOGRAPHY

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ABSTRACT

A simple radioisotopic method is described for the determination of glutamic acid decarboxylase in biological materials. It is based on the direct determination of γ-amino-butyric acid produced by the incubation of glutamic acid decarboxylase with the radiolabelled substrate. Separation is achieved by thin-layer chromatography on Dowex 1-acetate coated plastic strips. The assay is linear for GABA production with both time and enzyme concentration. The method was also used to determine the presence of glutamic acid decarboxylase in bovine subretinal intercellular fluid and retina.

INTRODUCTION

Glutamic acid decarboxylase (GAD) catalyzes the decarboxylation of L-glutamic acid to form γ-amino butyric acid (GABA). GABA has been recognized as a major inhibitory neurotransmitter in the mammalian central nervous system (1,2). GAD is also implicated in pathological states such as Parkinson's disease, Huntington's disease and schizophrenia (3-6).

In view of the significance of GAD in the central nervous system, there has been a great deal of interest in developing new assay methods based on the measurement of either of the products formed. However,

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none of the methods developed so far are easy or convenient for the determination of GAD in biological materials. The CO_2 evolution methods (7,8) are non specific, time consuming and not practical with large number of samples obtained during enzyme purification. On the other hand, the ion exchange column methods, based on GABA determination, (9-11) are specific and more sensitive than CO_2 evolution methods, but in general are lengthy, tedious and subject to interferences such as changes in pH or ionic strength. GAD determination based on the measurement of NADH formation after coupling GABA to GABA-transaminase and succinate semi-aldehyde dehydrogenase (12), is limited by the availability of enzymes in adequate purity and the effect of activators or inhibitors present in crude extracts on the coupling reaction. Recently, HPLC has also been used to determine GAD activity in biological materials (13,14).

In this paper, we report a simple, sensitive and specific microassay for determining GAD activity in crude brain and retinal extracts. The method is based on the separation of GABA from glutamic acid by thin-layer chromatography (TLC). We have also used this technique to look for the presence of GAD in subretinal intercellular fluid which separates neural retina from retinal pigment epithelium.

EXPERIMENTAL

Materials

L-[3,4-^{3}H]-glutamic acid, (40 Ci/mmole) was obtained from New England Nuclear (Boston, MA, USA). γ-Amino-[2,3-^{3}H]-butyric acid (60 Ci/mmole) was obtained from Amersham (Arlington Heights, IL, USA). ATP, unlabelled glutamic acid, GABA, α-ketoglutaric acid and succinic acid

were obtained from Sigma (St. Louis, MO, USA). Ethyl acetate was obtained from Eastman Kodak, (Rochester, N.Y.). Plastic sheets precoated with Ionex SB-Ac were obtained from Brinkmann (Westbury, NY, USA). Prior to use, 1.2 x 9.0 cm strips were cut and equilibrated with 0.05% acetic acid for 30 min as recommended by Devenyi (15).

Preparation of Crude Homogenate

Bovine brain and retina were homogenized with 20mM potassium phosphate, pH 6.8, 10mM mercaptoethanol and 1mM EDTA. The homogenates were sonicated for one min and centrifuged at 26,000 x g for 60 min at 4°C. The supernatant obtained in each case was used as the enzyme source. Bovine subretinal intercellular fluid was provided by Dr. Y. Lai (University of St. Louis) and was used without any further treatment.

Enzyme assay and thin-layer chromatography

The incubation mixture (total volume 50 µl) contained 50 mM potassium phosphate, pH 6.8, 1mM EDTA, 0.5mM pyridoxal phosphate, 5mM [^{3}H]-glutamic acid (800-2,000 cpm/nmole). The reaction was initiated by the addition of enzyme, incubated at 37°C, and stopped by rapid cooling in an ice bath. Immediately after cooling, 5 µl of the incubation mixture was spotted as a streak one cm from the bottom of the strip and dried. Ascending TLC was carried out using ethyl acetate/water (8:92). After the solvent had migrated about seven cm from the origin, the strips were removed and dried. Reference strips were also run with unlabelled glutamic acid and GABA under identical conditions. Spots corresponding to glutamic acid and GABA were cut and placed in scintillation vials. In cases where reference strips were not run, the strips were cut into one cm pieces and placed in separate vials.

Amino acids were eluted from the resin by adding 3.0 ml of scintillation fluid (ACS) and 0.5 ml of 20% formic acid as recommended by Himoe and Rinne (16). Recovery of radiolabelled glutamic acid and GABA from the strip using this method was 81 ± 2% (mean ± S.D., n=5), and 83 ± 3% (mean ± S.D., n=5), respectively.

Other methods

Protein was determined by the dye binding method using bovine serum albumin as a standard (17). The α-keto acids were detected by UV absorbance, and the pyridine - acetic anhydride staining method (18). GAD determination by reversed-phase HPLC was performed as described (13).

RESULTS

Separation of Glutamic Acid and GABA

Figure 1 shows the separation of unlabelled glutamic acid from GABA with ethyl acetate/water (8:92) obtained in 30 min. The contents of the assay mixture do not significantly affect the separation of glutamic acid and GABA. In three different runs, the R_F values for glutamic acid and GABA using this solvent system were found to be 0.06 ± 0.01 and 0.95 ± 0.02, respectively. The inclusion of 0.05% acetic acid in the solvent mixture does not affect the mobility of GABA, while the R_F value of glutamic acid increased to 0.09. On the other hand, inclusion of 1% pyridine in the solvent system did not change the R_F values of either glutamic acid or GABA. Also, the presence of 1% trichloroacetic acid in the assay mixture does not interefere with the migration of glutamic acid and GABA. Thus, the system appears to be insensitive to pH.

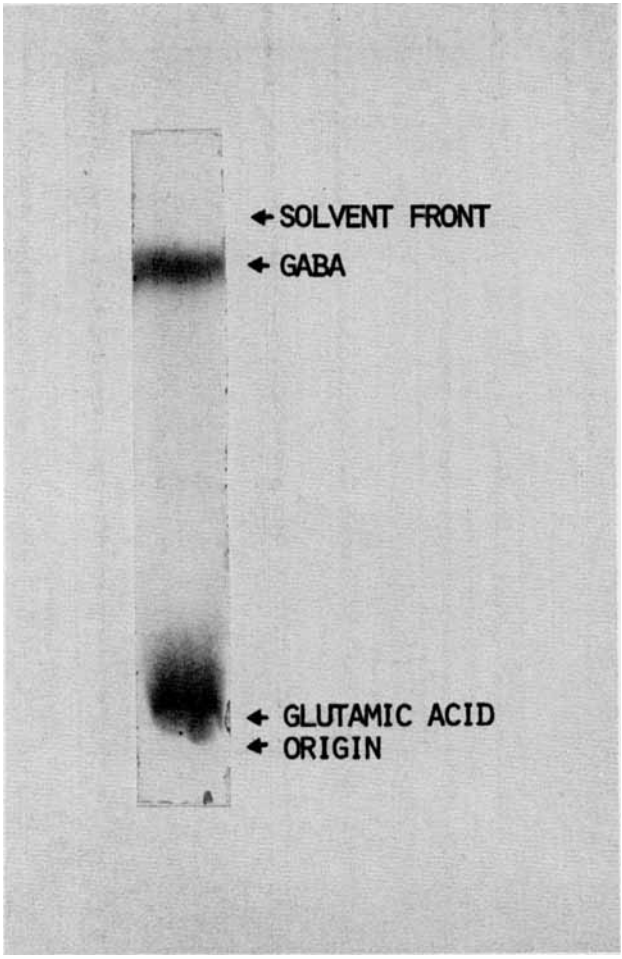

FIGURE 1: Separation of glutamic acid and GABA. A 2 µl aliquot containing 4 µg of glutamic acid and GABA in 50 mM phosphate buffer, pH 6.8 was spotted. After development, the strip was dried, sprayed with 0.2% ninhydrin in butanol/acetic acid (95:5) and spots visualized after brief exposure at 70°C.

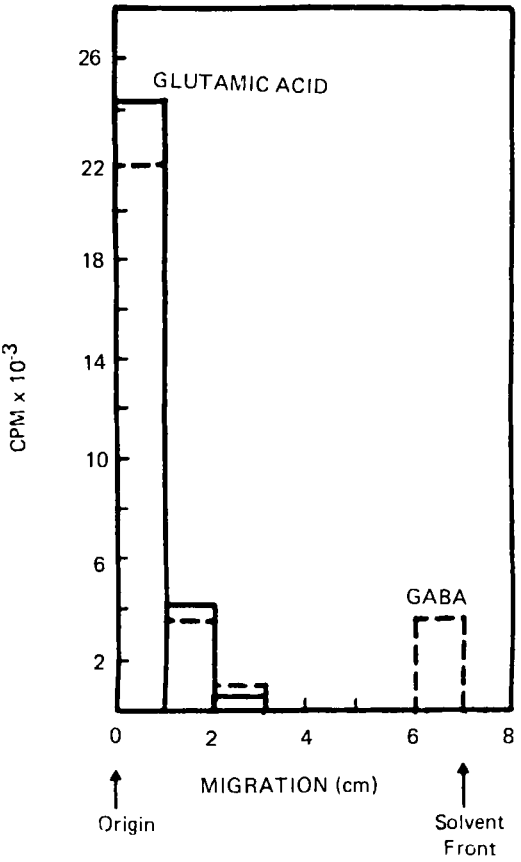

FIGURE 2: Separation of [^{3}H] GABA (---) from [^{3}H]-glutamic acid. After development, the strip was dried, cut into one cm pieces and radioactivity counted as described in the text. The assay mixture was incubated with 180 µg of crude enzyme preparation at 37°C for 60 min. The control (——) contained the assay mixture mixed with enzyme at 37°C followed immediately by cooling to 0°C. The composition of the assay mixture was the same as described in Materials and Methods.

Figure 2 shows the separation of GABA formed from radiolabelled glutamic acid by the GAD present in crude brain homogenate. Glutamic acid remained near the origin, and GABA formed by the enzymatic reaction moved near the solvent front resulting in a complete separation of the two components. No significant amount of GABA was formed during the time when the enzyme assay mixture was immediately cooled and spotted. In order to insure the elimination of non enzymatic formation of GABA, the radiolabelled glutamic acid was incubated at 37°C for 2 h with the assay mixture except that the addition of enzyme was omitted. As shown in Figure 3, the chromatogram revealed only the presence of glutamic acid and there was no formation of GABA when the enzyme was eliminated from the assay mixture.

Relationship of GABA Synthesis with Time and Protein Concentration

A crude enzyme preparation of bovine brain was used to test the linearity of the assay with time and protein concentration. The linear relationship of GABA synthesized with respect to time is shown in Figure 4A and has a correlation coefficient of 0.994. The amount of GABA synthesized was also linear with respect to protein concentration of crude brain extract (Figure 4B) and has a correlation coefficient of 0.997.

Levels of GAD in Retina and Subretinal Fluid

The assay described above was used to look for the presence of GAD in subretinal intercellular fluid and retina. As shown in Table 1, retina revealed significant GAD activity, while subretinal intercellular fluid showed negligible activity. The production of GABA was dependent on the amount of crude retinal extract used. The specific activity of the enzyme was determined to be 0.108 ± 0.005 from three different experiments.

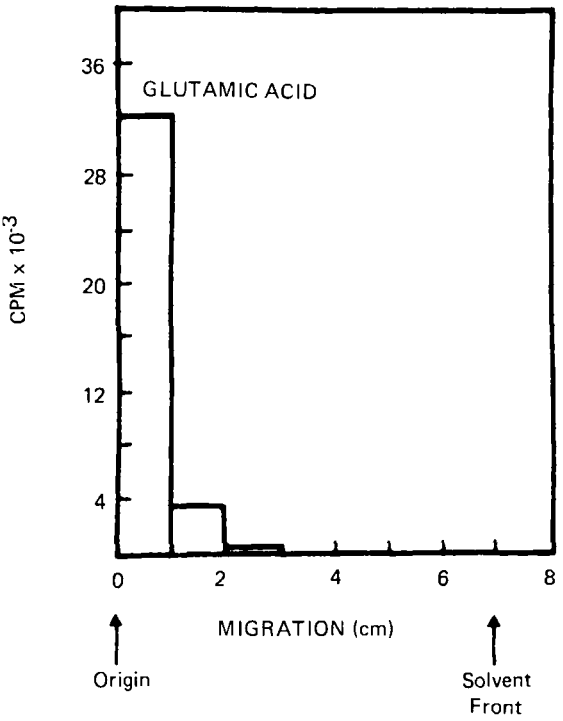

FIGURE 3: Migration of [^{3}H] glutamic acid. The assay mixture without enzyme was incubated with the radiolabelled substrate at 37°C for 2 h. The composition of the assay mixture was the same as described in Materials and Methods.

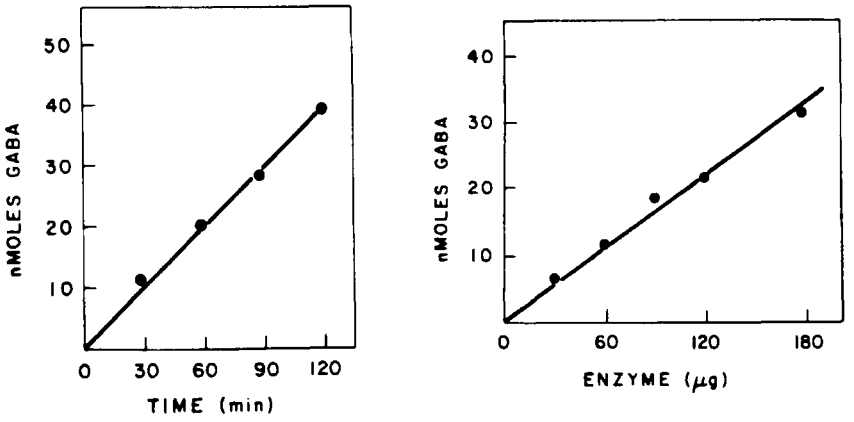

FIGURE 4: Linear relationship of GABA synthesis with time and protein concentration. A) Assay performed at 37°C with 96 μg of crude enzyme protein for various incubation times. B) Assay performed at 37°C for 60 min with different concentrations of enzyme. For additional experimental details, see Materials and Methods.

TABLE 1

Levels of GAD in Bovine Retina and Subretinal Intercellular Fluid

Tissue	Protein Conc.	nMoles of GABA Formed/h	Specific Activity
Retina	300 μg	32.50	0.108
Retina	300 μg	31.02	0.103
Retina	450 μg	51.20	0.113
Subretinal Inter-Cellular Fluid	300 μg	1.25	0.004

Specific activity is defined as μ moles of GABA formed/h/mg protein.

DISCUSSION

Determination of GAD by TLC offers many advantages over other similar methods such as high voltage paper electrophoresis (19,20) ion exchange chromatography (9-11) or HPLC (13,14). The identity of GABA as the only product formed under the present GAD assay conditions has been determined by reversed-phase HPLC (13). Also, the formation of non-specific metabolites such as α-ketoglutarate or succinate as observed in non-neural tissues (21) does not interfere with the present assay. Under identical conditions these metabolites move slow (R_F, 0.02) in comparison to GABA (R_F, 0.95).

The present method measures GABA formation directly and is simpler to use than ion exchange chromatography or high voltage paper electrophoresis. The enzyme activity determined by this method in brain or retinal homogenates was equivalent to the activity obtained by reversed-

phase HPLC (13). This assay is also more economical and sensitive than the CO_2 evolution method since the present method uses tritium labelled glutamic acid. Under the present conditions of assay, it is possible to detect GAD activity present in 1 μg of crude extract using tritium labelled glutamic acid (2,000 cpm/nmole). Also, unlike ion exchange chromatography, separation of glutamic acid from GABA by the present TLC method is not affected by changes in pH or ionic strength of the assay mixture.

In summary, we feel the assay is sensitive, simple, specific, rapid and multiple samples can be run under identical conditions. This method should be useful in determining low concentration of enzyme as ordinarily found in cells in culture and in studying the effect of various metabolites on the specific production of GABA by the GAD reaction.

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