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USE OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR SIMULTANEOUS DETERMINATION OF GLUTAMINE SYNTHETASE AND GLUTAMIC ACID DECARBOXYLASE IN CRUDE EXTRACTS

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SUMMARY

Glutamine and γ-aminobutyric acid (GABA), formed from glutamic acid in crude tissue extracts by glutamine synthetase and glutamic acid decarboxylase respectively, were separated by derivatization with dansyl chloride followed by reversed-phase high-performance liquid chromatography on an Altex Ultrasphere ODS-5 column. The mobile phase was a gradient of 100 m*M* potassium dihydrogen phosphate (pH 2.1) with 0—40% acetonitrile. The amounts of glutamine and GABA formed from glutamic acid were determined under different reaction conditions.

INTRODUCTION

Glutamine and γ-aminobutyric acid (GABA) are formed from glutamic acid by glutamine synthetase (GS) and glutamic acid decarboxylase (GAD) respectively. The presence of both enzymes has been demonstrated in neuronal as well as non-neuronal systems. GABA has been assumed to be a major inhibitory neurotransmitter in the central nervous system [1, 2]. Glutamine occupies a central position in nitrogen metabolism since it serves as a source of nitrogen for various metabolites, which in turn are used for the formation of proteins, carbohydrates and nucleic acids [3, 4]. The interest in our laboratory is aimed at differential metabolism of glutamic acid by retinal cells under various conditions. Recently, Pishak and Phillips [5] utilized a double column method to separate glutamine and GABA from glutamic acid. This was necessitated because under their assay conditions for GS, both glutamine and GABA were formed. To achieve resolution of the three components, this method requires two ion-exchange columns.

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Reversed-phase high-performance liquid chromatography (HPLC) offers advantages over conventional chromatographic techniques in both resolving power and time. We utilized this technique to resolve glutamic acid, GABA and glutamine from each other after derivatization with dansyl chloride. In addition, we present evidence that under proper assay condition, glutamine is the only product formed by the GS reaction and GABA is the only product formed by the GAD reaction.

MATERIALS AND METHODS

Reagents

Acetone, distilled in glass and acetonitrile, UV grade, distilled in glass, were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Glutamic acid, GABA, glutamine, dansylated derivatives of these amino acids, and ATP were obtained from Sigma (St. Louis, MO, U.S.A.). [3,4-^{3}H]Glutamic acid (40 Ci/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). Potassium dihydrogen phosphate, analytical grade, was obtained from Gallard-Schlesinger Chemical Corp. (Carle Place, NY, U.S.A.). Concentrated phosphoric acid, AR, was obtained from Mallinckrodt (St. Louis, MO, U.S.A.). Distilled water was further purified by passing through a Milli-Q system (Millipore, Bedford, MA, U.S.A.) followed by glass distillation. Dansyl chloride for derivatization was prepared fresh daily by diluting the stock solution (10% in acetone obtained from Pierce (Rockford, IL, U.S.A.) with acetone to yield a final concentration of 0.25%. Sodium bicarbonate buffer (100 m*M*, pH 9.5) was passed through 0.45-μm filter (Millipore). All other chemicals were reagent grade and obtained from the usual sources.

Enzymes source

Crude enzyme preparations were obtained by homogenizing bovine retina and brain in three volumes of 25 m*M* imidazole buffer (pH 7.1), 10 m*M* mercaptoethanol and 0.1 m*M* EDTA. The homogenate was centrifuged at 1000 *g* to remove nuclei and cellular debris. The supernatant was withdrawn carefully and used as a source of GS and GAD. GS was also purified from retina by column chromatography and was judged to be homogeneous on the basis of electrophoretic techniques and immunodiffusion. The details of purification procedure and characterization will be described elsewhere [6].

Enzyme assays

The glutamine synthetase assay was carried out in a 50-μl aliquot (pH 7.4) containing 50 m*M* imidazole, 4 m*M* NH_4Cl, 10 m*M* ATP, 20 m*M* $MgCl_2$, 0.2 m*M* glutamic acid and 1—2 μCi of [^{3}H]glutamic acid. After addition of enzyme, the mixture was incubated at 37°C for 15 min and the reaction was stopped by rapidly cooling in an ice bath. A 20-μl aliquot was withdrawn and vortexed with 20 μl of 1 *N* acetic acid. The insoluble material was removed by centrifugation in a microfuge for 5 min. A 20-μl aliquot of the supernatant was withdrawn into a PTFE-lined capped sample vial, lyophilized and used for dansylation.

The buffer used for the GAD assay was 100 m*M* potassium phosphate (pH

6.8), 0.5 m*M* pyridoxal phosphate, 1 m*M* EDTA. The reaction was initiated by the addition of crude enzyme extract to 100 μl of buffer, 2 μl glutamic acid (5 m*M*) and 1—2 μCi of [^{3}H]glutamic acid. After incubation at 37°C, the reaction was stopped by rapidly cooling in ice. A 50-μl aliquot was withdrawn and the pH adjusted between 9 and 9.5 by the addition of 0.1 *N* sodium hydroxide. The insoluble material was removed by centrifugation in a microfuge for 5 min. A 50-μl aliquot was withdrawn into a PTFE-lined capped sample vial, lyophilized and used for dansylation.

Dansylation

Dansylation was carried out by a modification of the method of Gray and Hartley [7]. The lyophilized material in the sample vial was mixed with 50 μl of the bicarbonate buffer and 100 μl of the working dansyl chloride solution. The vial was capped and the contents were mixed thoroughly. After incubation at 70°C for 15 min in a water bath, the vial was removed, cooled in ice for about 5 min and 20 μl were injected into the HPLC column.

Chromatography

A Beckman Model 332 gradient liquid chromatograph equipped with a Beckman Model 210 sample injection valve with a 20-μl loop and an LKB UV monitor operating at 206 nm was used. An Altex Ultrasphere reversed-phase ODS-5 column (250 × 4.6 mm) was used. The mobile phase was a gradient of 100 m*M* potassium dihydrogen phosphate pH 2.1 with 0—40% acetonitrile. The flow-rate was 1 ml/min at ambient temperature. Fractions (0.5 ml) were counted in 2 ml of ACS scintillation fluid.

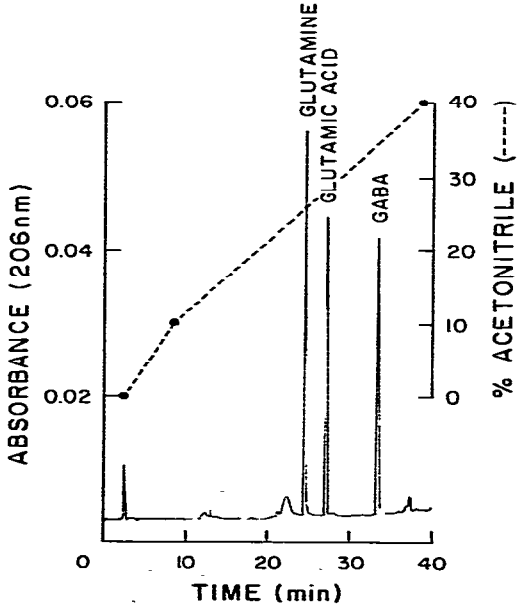

Fig. 1. Chromatogram showing the separation of 0.5 μg of dansylated glutamine, glutamic acid and GABA. The dashed line indicates the mobile phase gradient (% acetonitrile) used.

Protein determination

Protein was measured in all samples by the dye binding method [8] using bovine serum albumin as a standard.

RESULTS

The chromatogram showing the separation of dansylated glutamic acid, GABA and glutamine standards along with the gradient used can be seen in Fig. 1. The separation of these three compounds was reproducible, elution times were within ± 1%. As can be seen from the chromatogram well resolved peaks were obtained. Glutamine elutes at 25 min (flow-rate 1 ml/min), glutamic acid elutes at approximately 27.5 min, and GABA elutes at 33.5 min. Undansylated glutamic acid, glutamine and GABA are not separated from each other significantly and elute early. Prior dansylation also increases the sensitivity of detection of the amino acids by UV absorption and fluorescence. It is possible to detect as little as 100 ng of each amino acid by UV absorption at 206 nm and even less can be observed with a fluorescence detector.

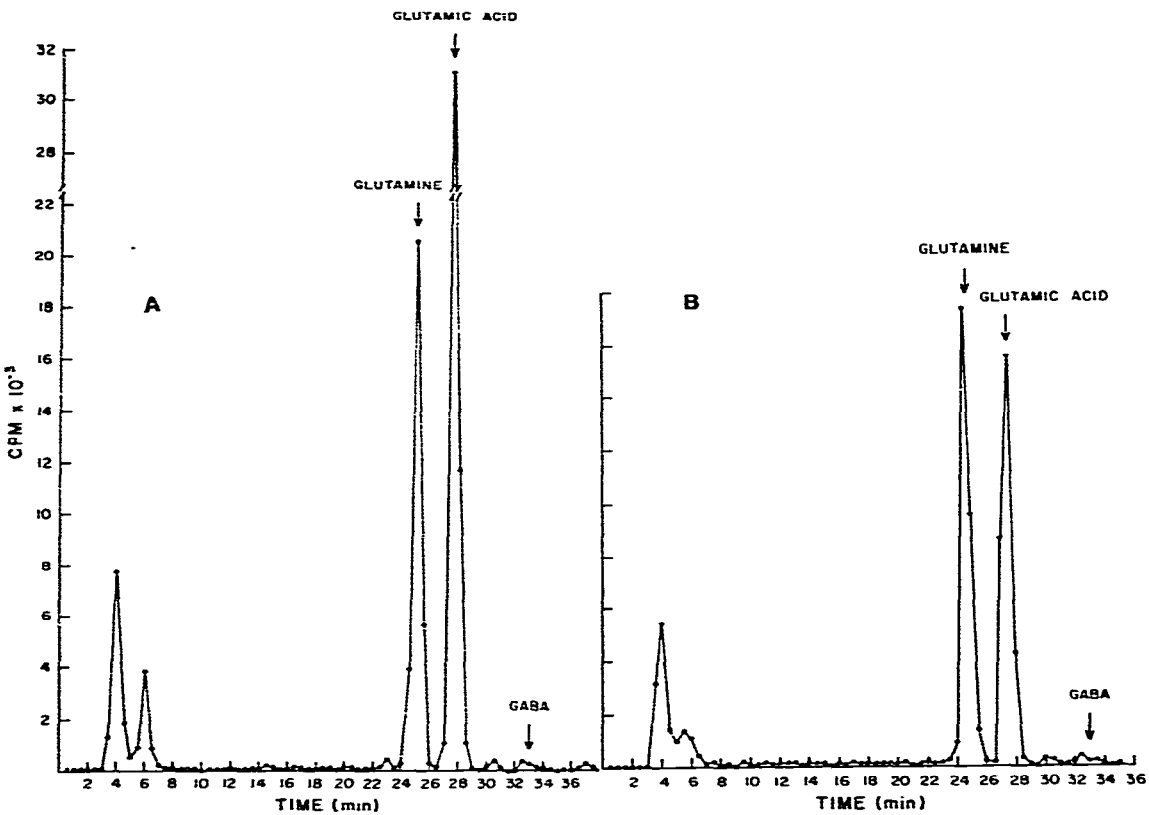

Fig. 2. Chromatograms of the reaction mixture using (A) purified retinal glutamine synthetase, 0.28 μg of purified enzyme protein plus assay mixture was incubated for 45 min; (B) 0.24 mg protein of crude retinal extract under GS assay conditions. For further details see Materials and Methods.

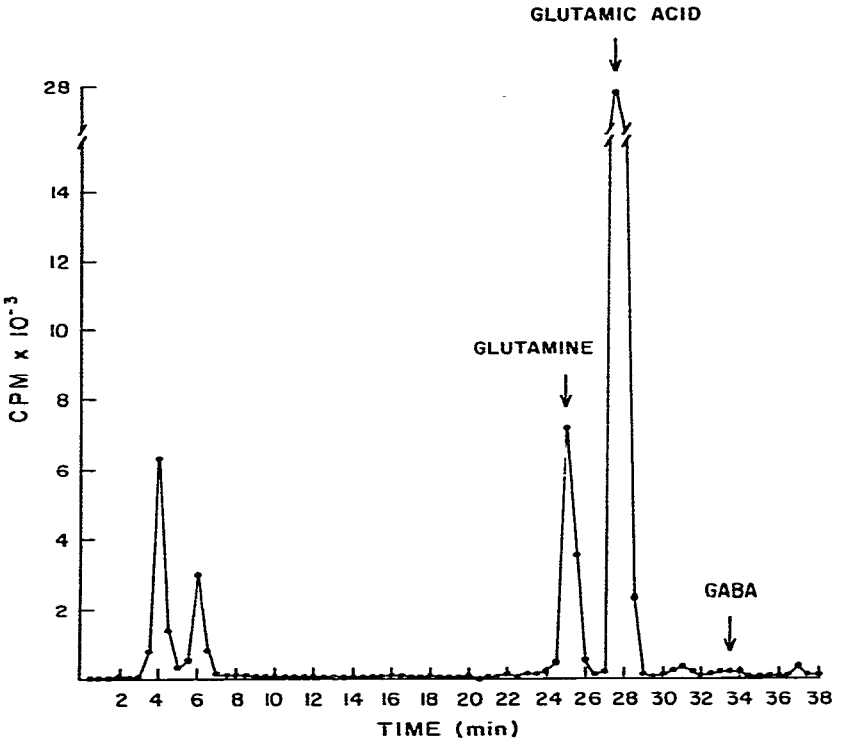

Fig. 3. Chromatogram of the reaction mixture using 0.32 mg of crude brain extract under GS assay conditions. For further details see Materials and Methods.

Two major peaks corresponding to glutamine and glutamic acid are observed for the reaction catalized by purified retinal GS and crude retinal homogenate (Fig. 2A and B). The two early peaks correspond to undansylated material. The control run of [^{3}H]glutamic acid after dansylation also revealed the presence of two early peaks besides a major peak for glutamic acid. The second early peak (6 min) was found to be a contaminant (2—4%) in our tritiated glutamic acid which would not dansylate. At the present time the identity of the peak is not known; however, due to its low concentration it appears to have no effect upon the results. With this method, it is possible to detect enzyme activity in a small amount of biological material having only 2 μg of protein. The amount of GABA found in the glutamine synthetase assay was less than 1% and so for all practical purposes no GABA is formed. Similar results were obtained using crude brain extract (Fig. 3) under the same GS assay conditions. In this case, however, lower enzyme activity resulted in a smaller glutamine to glutamate ratio. Again, no GABA was seen. When the experiment with crude brain extract was carried out without the addition of ATP or Mg^{2+}, (Fig. 4), there was no significant formation of glutamine. If, however, the reaction with the crude brain or retinal extract was carried out with pyridoxal phosphate but no ATP or Mg^{2+} (GAD assay conditions), GABA was formed but there was no significant formation of glutamine (Fig. 5A and B). With this method, it is possible to detect GAD in biological material having only 10 μg of protein. The percent dansylation from nine separate experiments was found to

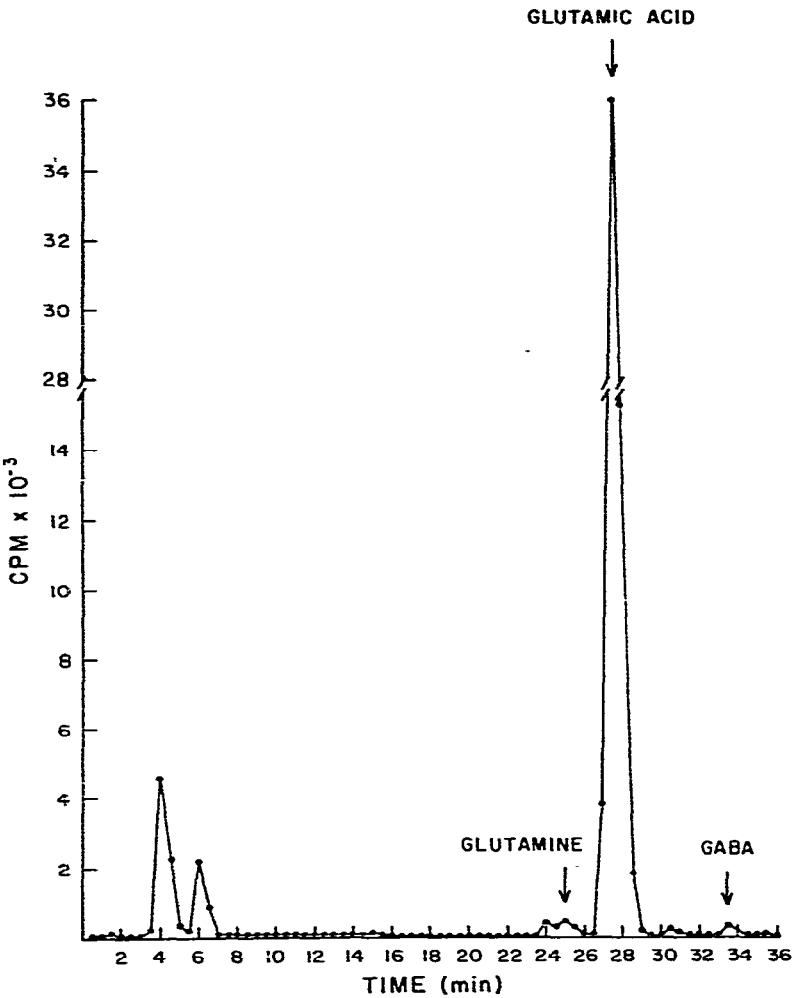

Fig. 4. Chromatogram of the reaction mixture of crude brain extract under GS assay conditions without ATP and Mg^{2+}. Other conditions were the same as in Fig. 3.

be 85.64 ± 3.52%. Recovery of amino acids from the column was 100% and no amino acid was detected after final wash with acetonitrile. Table I summarises the results of glutamine and GABA formation under various conditions by crude retinal and brain extracts.

DISCUSSION

Reversed-phase HPLC has been shown to be a powerful tool for studying the separation of amino acids [9] and therefore should be equally valuable in studying the metabolism of amino acids and their metabolites in cultured cells under different conditions. We have been interested in the metabolites, glutamine and GABA, formed from glutamic acid. At present the only method of studying these metabolites is by the use of two separate anion-exchange columns [5]. Therefore we developed a reversed-phase HPLC method for the

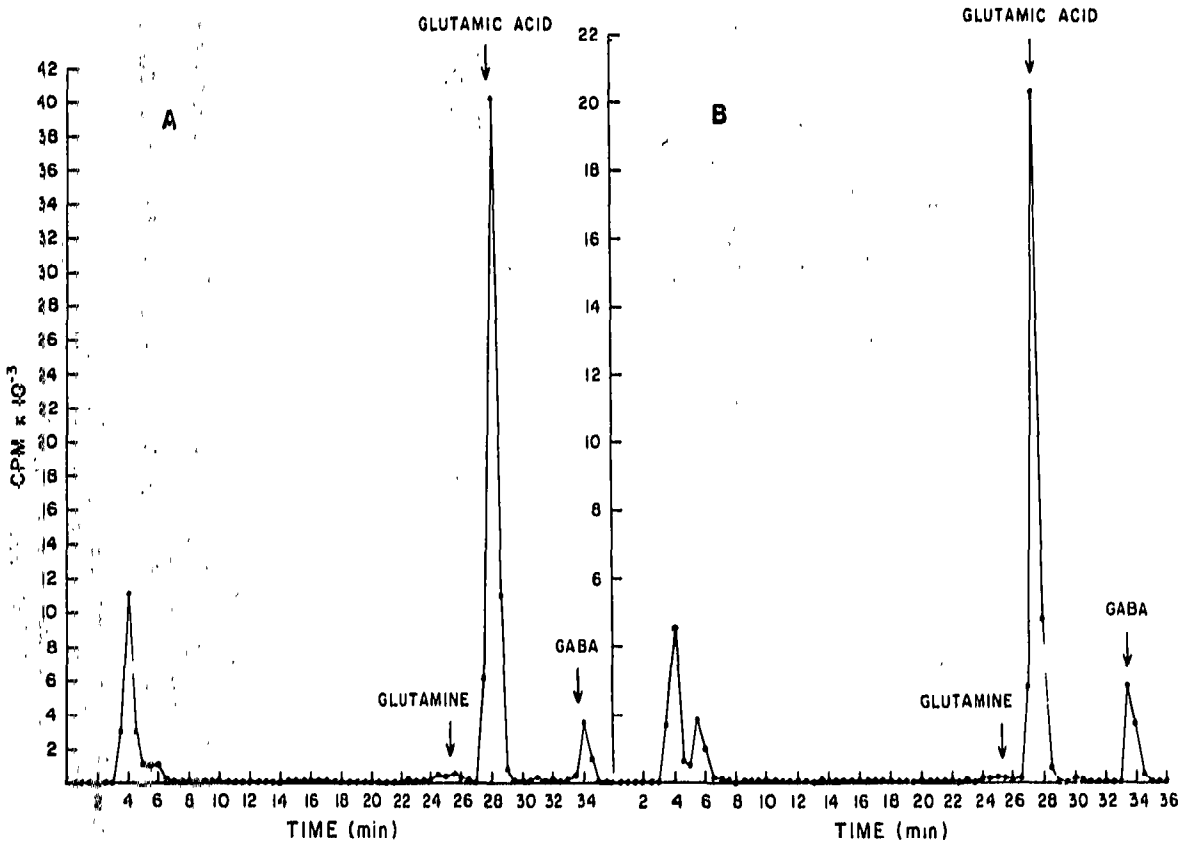

Fig. 5. Chromatograms of the reaction mixture of (A) crude brain extract under GAD assay conditions, 0.17 mg of crude enzyme protein plus assay mixture were incubated for 1 h; (B) crude retinal extract under GAD assay conditions, 0.30 mg of crude enzyme protein plus assay mixture were incubated for 2 h. For further details see Materials and Methods.

TABLE I

PERCENTAGE CONVERSION OF GLUTAMIC ACID BY RETINA AND BRAIN UNDER GS AND GAD ASSAY CONDITIONS

Tissue	Assay conditions	GABA (%)	Glutamine (%)
Retina*	GS assay	<1	50
Brain**	GS assay	<1	17
Brain***	GS assay, —ATP, —Mg^{2+}	<1	1.4
Retina§	GAD assay	15	<1
Brain§	GAD assay	8.3	<1

*Same conditions as Fig. 2B.
**Same conditions as Fig. 3.
***Same conditions as Fig. 4.
§ Same conditions as Fig. 5.

separation of these three compounds. This method can be utilized to determine the products formed by both GS and GAD. As seen in Table I, using crude enzyme preparations under proper assay conditions, either glutamine or GABA is the principal metabolite of glutamic acid. As shown here and by others [10, 11], ATP and Mg^{2+} are required as cofactors for glutamine synthetase and virtually no reaction takes place in their absence. On the other hand, ATP and Mg^{2+} are inhibitory for GAD [12, 13]. Thus, by simply varying the assay conditions one can detect either GABA or glutamine as the product from glutamic acid while using whole cell preparations where both reactions might occur. We are utilizing this method at present to study different metabolic routes of glutamic acid in retinal cells.

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