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REVIEW

CHROMATOGRAPHIC ANALYSIS OF GLUTAMIC ACID DECARBOXYLASE IN BIOLOGICAL SAMPLES*

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CONTENTS

1. INTRODUCTION

L-Glutamic acid (GA) is formed from the deamidation of L-glutamine by the enzyme L-glutamine aminohydrolase (EC 3.5.1.2., glutaminase) [1, 2]. The product of this reaction is then α-decarboxylated by L-glutamic acid decarbox-

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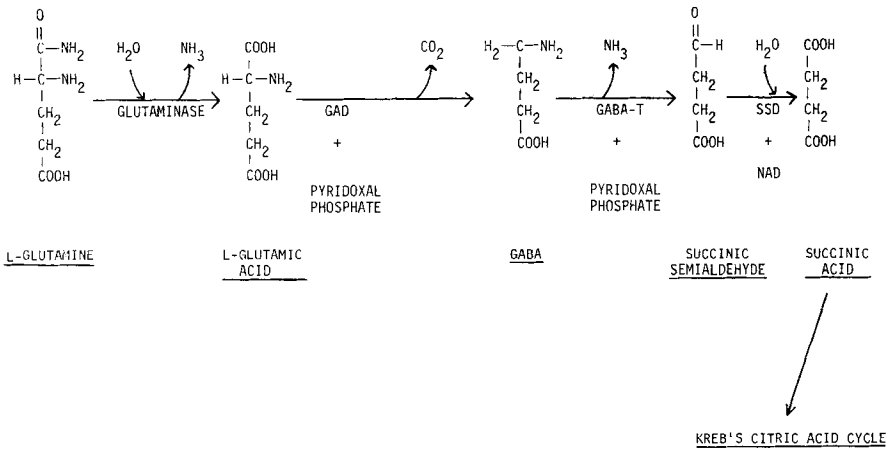

Fig. 1. Overall reaction scheme for conversion of L-glutamine to GABA and its metabolism to succinic acid. The enzyme GAD is believed to be the rate-limiting step which produces steady-state levels of GABA.

ylase (EC 4.1.1.15., GAD) to form γ-aminobutyric acid (GABA). GAD is a highly specific enzyme that requires pyridoxal phosphate as a coenzyme and is believed to be the rate-limiting step that produces steady-state concentrations of GABA in tissues [3—5]. GABA is metabolized by 4-aminobutyrate-2-ketoglutarate transaminase (EC 2.6.1.19., GABA-T) to succinic semialdehyde and further by succinic semialdehyde dehydrogenase (EC 1.2.1.16., SSD) to succinic acid which can enter the Krebs citric acid cycle. This overall scheme of the reactions is presented in Fig. 1. An excitatory effect is observed by GA in the central nervous system (CNS) whereas its α-decarboxylation product (GABA) is established as a principal inhibitory neurotransmitter in the CNS of vertebrates [6—8]. GABA is considered as a hyperpolarizing inhibitory neurotransmitter and lately data indicate GABA mediated depolarization of afferent nerve terminals suggesting a presynaptic inhibitory function [9—11].

GAD is indicated to be the rate-limiting enzyme that determines steady-state levels of GABA in the nervous system. The concentration of GABA correlates well with the GAD activity in tissues. However, GAD is found solely within the neurons whereas GABA is distributed in glial cells as well as within neurons. Therefore, the activity of this enzyme is used as a more precise marker for GABAnergic neurons than GABA due to the latter's distribution and possible metabolism during tissue preparation [12].

In the CNS, GABA projections from cortex to neostriatum to globus pallidus (GP), entopeduncular nucleus (EP) and substantia nigra (SN) [13, 14] as well as from SN to ventromedial thalamus (VM) [15] are reported. In structures associated with the extrapyramidal system high concentrations of GABA and GAD are observable. In several mammalian species the highest levels of these two components are found in the SN and GP [16, 17].

Assay procedures for this enzyme and its major product have their greatest utility in clinical practice for postmortem studies of CNS pathology. Reduced levels of GABA are found in brain regions of some patients with schizophrenia [18] and dominantly inherited cerebellar disorders [19]. Postmortem brains of

Huntington's chorea patients (these patients presently account for 1% of all chronically hospitalized individuals in mental institutions) contain reduced content of GABA and GAD in cortex, striatum and SN [20—23]. Cerebral spinal fluid from live Parkinson's disease patients contains significantly decreased quantities of GABA when compared with normal controls [24] and published autopsy studies present data of low concentrations of GAD and GABA in striatum and SN [20, 25]. Recently, however, Perry et al. [26] have published data that mean GAD activity in Parkinson's disease patients (when matched with better control subjects for agonal status) is lower in the putamen than that of controls, but that the difference is not significant; also, increased levels of GABA are found. This recent study cast doubt on the hitherto traditional assumption that GABAnergic neurotransmission is decreased in the striatum of Parkinson's disease [26].

To date, there are four different types of procedures utilized for analysis of GAD in tissue samples. Of these methods only one directly measures the enzyme's concentration whereas the others measure the enzymatic activity. The first technique is that of mannometric determination of carbon dioxide (CO_2) evolved by α-decarboxylation of GA. Subsequently, via use of L-[^{14}C]GA as substrate, radiochemical methods are now employed for measurement of $^{14}CO_2$ evolution with picogram sensitivities. The second procedure consists of analysis of GABA formed by the coupled reactions of GABA-T and SSD which gives rise to an equivalent number of moles of NADPH. Fluorometric quantitation of NADPH by this enzymatic cycling process approaches the femtomole range. A third method utilizes the measurement of GABA formation from tissue homogenates incubated with GA as substrate. GABA is separated from other components in the mixture chromatographically and with the use of different analytical techniques the limits of detection range from nanograms to picograms. The last methodology requires the use of immunocytochemical and immunological techniques with nanogram quantitation. These procedures involve the direct measurement of the enzyme's concentration and not its activity. Antibodies are produced against GAD and its concentration is determined fluorometrically, radiochemically or microscopically.

In this article all four methodologies used for GAD analysis will be discussed. However, the major focus will be upon those that involve or rely upon chromatographic procedures for separation and analysis. For more detailed information concerning the other non-chromatographic procedures the reader is referred to the references of Roberts et al. [7], Wu [12] and Krogsgaard-Larsen et al. [27]. Chromatographic methods have become increasingly important for quantitation and specificity of analysis. Most involve the measurement of the enzyme's activity via GABA formation following incubation of the tissues. Also discussed in this review will be some of the common problems that arise and introduce errors in activity calculations such as alternative pathways and postmortem changes.

2. ASSAY ERRORS

2.1. Alternative pathways

In 1972, Wilson et al. [28] reported an observation of differences in GAD activity measurement between $^{14}CO_2$ evolution and [^{14}C]GABA formation in tissue cell cultures. The radiolabeled gas was analyzed by radiochemical methods and [^{14}C]GABA was separated from the cell culture mixture after incubation with [^{14}C]GA via thin-layer chromatography (TLC) and quantitated by scintillation counting. GAD activity measured by [^{14}C]GABA formation was as much as 10% less than that estimated by $^{14}CO_2$ production. It was concluded that excess $^{14}CO_2$ was produced from glutamate by pathways other than GAD. Drummond and Phillips [29] later published similar data concerning GAD activity in non-neural tissues. [^{14}C]GABA was separated and analyzed with an amino acid analyzer from crude tissue homogenates. The differences observed with greater $^{14}CO_2$ production than [^{14}C]GABA was attributed to the alternative CO_2 producing pathway of the coupled reactions of glutamic acid dehydrogenase (EC 1.4.1.2.) and lipoate acetyltransferase (EC 2.3.1.12.) of the α-ketoglutarate dehydrogenase complex.

Another problem that has arisen with CO_2 methods has been associated with impurities that have been found in commercially labeled GA. These impurities gave rise to additional CO_2 production without concurrent stoichiometric production of GABA [29—31]. It was noted that a carbonyl-trapping agent, aminooxyacetic acid, stimulated excess formation of $^{14}CO_2$ in brain homogenates when unpurified [^{14}C]GA was utilized [31]. However, upon purification of the substrate, significantly less $^{14}CO_2$ was formed. The impurity 2-pyrolidone-5-carboxylate isolated from commercial L-glutamate preparations has been suggested to cause increased CO_2 production [32]. Using impure radiolabeled L-glutamate, Wu et al. [33] analyzed GAD activity in various tissues by five different assay techniques. One method utilized the trapping of radiolabeled $^{14}CO_2$ whereas the other four involved the quantitation of radiolabeled GABA formation by various chromatographic means. In brain tissue all five techniques gave similar results; however, when samples of non-neural tissues were tested (heart, liver, kidney) only the methods utilizing GABA formation for activity determination had similar results. The activity measured via the $^{14}CO_2$ technique was three to four times greater than that of the other procedures and lead the authors to suggest that GAD measurement in non-neural tissue did not give valid information for activity measurements by CO_2 trapping techniques.

Wu et al. [34] demonstrated that mouse brain GAD will also utilize L-aspartic acid as a substrate and identified the α-decarboxylation products as β-alanine. This compound possessed approximately 3—5% of the activity of L-glutamate as substrate. Also, recent evidence suggests that GABA may be produced from 4-aminobutyraldehyde in mammalian brain by 4-aminobutyraldehyde dehydrogenase [35]. Therefore, it is possible to have GABA production from alternative pathways. However, at this time information is limited and further evidence must be acquired before any conclusive statements can be made concerning alternate GABA producing pathways.

2.2. Postmortem changes

Extremely rapid GABA formation is observed in rat brain tissues if it is not frozen within minutes of death [36—41]. The increase in GABA formation begins about 1—2 min following death and reaches levels of 140% of initial values within 5 min [36, 37]. These increases in GABA concentration occur in both solid tissue or tissue suspensions that are not cooled immediately postmortem. A possible explanation for these observed increases in GABA content is that there is a 9% increase in the activity of the GAD enzyme in rats for the first 2 h after decapitation followed by a slow decline to zero activity. After a period of time the GA content would be expected to be reduced and not as much GABA, as is found experimentally, produced. However, hydrolysis of proteins, peptides and reduced glutathione may serve as a possible source of GA [42]. Also, the degradative enzyme of GABA, GABA-T, apparently loses its activity for metabolism of GABA and compounds such as aminooxyacetic acid have been demonstrated to specifically inhibit GABA-T and not GAD in rat brain tissue [36]. In Table 1 are presented data [38] of rapid postmortem changes observed fluorometrically in temperature preserved and non-preserved rat brain tissue. The left hemispheric samples were immediately cooled postmortem whereas the right hemisphere samples were allowed to stand at room temperature (22°C) for 10 min before being treated for analysis. In the GP and SN (medial and lateral) over 100% increases in GABA were noted in the samples left at room temperature.

TABLE 1

POSTMORTEM CHANGES IN GABA OBSERVED IN LEFT AND RIGHT HEMISPHERES OF RAT BRAIN

Values expressed as μg GABA.

Brain region	Left hemisphere*	Right hemisphere**
GP	0.64	1.39
SN medial	2.45	5.12
SN lateral	2.07	4.28

*Samples immediately cooled postmortem.
**Samples left at room temperature 10 min postmortem.

A number of techniques have been developed to preserve the enzyme's activity and reduce the rapid postmortem GABA increases. These techniques include such methods as dropping rats head first into liquid nitrogen or decapitation followed by liquid nitrogen treatment or ice water [36, 37]. A method reported by Holdiness et al. [43] has proved useful for analysis of GAD activity in micropunches (< 2 mg) of rat brain tissue. In this procedure the rat was decapitated with a guillotine and the brain rapidly removed and frozen in dry ice. The brain samples were transferred to a temperature controlled microtome where micropunches were obtained of appropriate slices. Since GABA production was analyzed for the enzyme's activity, the sample was homogenized and half was transferred to a test tube containing 10% trichloroacetic acid for en-

zyme inactivation. This sample served as the blank and its concentration of GABA was subtracted from that of non-denatured incubated sample to obtain the correct GAD activity.

3. ASSAY PROCEDURES (NON-CHROMATOGRAPHIC)

3.1. Mannometric and radiolabeled carbon dioxide methods

Roberts and co-workers [4, 44] developed the first mannometric technique for GAD activity analysis which involved the measurement of CO_2 evolution following α-decarboxylation of GA. In 1959 Albers and Brady [45] reported the first radiometric procedure utilizing L-[1-^{14}C] GA as substrate leading to the formation of $^{14}CO_2$. The limit of detection of this particular method was 10^{-11} mol of radiolabeled carbon dioxide. Later Sisken et al. [46] and Roberts and Simonsen [47] made improvements in gas-trapping procedure and apparatus used for gas collection. It was noted that the atmosphere in which the crude homogenates were incubated could effect the results of the assay. Sisken et al. [46] reported that higher concentrations of $^{14}CO_2$ were recorded in an oxygen atmosphere as opposed to a nitrogen atmosphere. Others have also observed increased CO_2 production in an oxygen containing atmosphere as compared with inert atmospheres even after impurities had been carefully removed from commercial preparations of GA [47, 48]. This excess production of $^{14}CO_2$ was greatly reduced when an inert atmosphere, Triton X-100, or mitochondrial electron transport inhibitors were utilized during incubation of the homogenates [48, 49].

3.2. GABA-transaminase and fluorometric procedures

In Fig. 2 the enzymatic cycling procedure of the coupled reactions of GABA-T and SSD is presented [12, 50]. This reaction scheme combines the GABA assay of Jakoly and Scott [51] with the enzymatic cycling procedure of Lowry et al. [52]. GABA and α-ketoglutarate are converted to succinate semialdehyde (SSA) and glutamate by GABA-T and this product (SSA) is further converted to succinate in a second reaction by succinate semialdehyde dehydrogenase. Equivalent moles of $NADP^+$ in the second reaction are reduced to NADPH. In the third step of the reaction NADPH is oxidized to $NADP^+$ by glutamate dehydrogenase (GDH) and then reduced back to NADPH by glucose-6-phosphate dehydrogenase (EC 1.1.1.49., G-6-PD) in the fourth reaction. An amplification of 10,000 fold is achieved in 1 h. Therefore, for every GABA or NADPH molecule added, 10,000 molecules of 6-phosphogluconate are formed. The 6-phosphogluconate (6-PG) in reaction 4 is converted to ribose-5-phosphate (R-5-P) by 6-phosphogluconate dehydrogenase (EC 1.1.1.44., 6-PGD) and $NADP^+$ is subsequently reduced to NADPH which is determined fluorometrically. The production of NADPH is equivalent to the amount of GABA formation. Amplifications up to 100,000,000 are possible with a detection limit of 10^{-18} mol GABA [12]. This technique can be applied to the analysis of GAD activity in single isolated nerve cell bodies in the CNS [53]. However, this method is limited in crude extracts by the effects of inhibitors or activators on

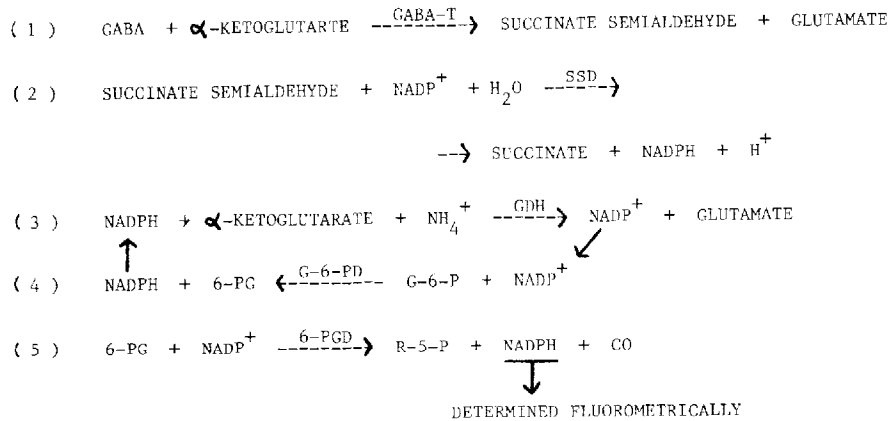

Fig. 2. Principle of microdetermination of GABA by a combination of enzymatic cycling and GABAase system (GABAase refers to the coupled enzymes: GABA-T and SSD). (Reproduced with permission from ref. 12.)

the coupling reactions and by the inadequate purity of available enzymes.

The first fluorometric procedure was reported by Lowe et al. [54] in 1958. This method relied upon measurement of GABA formation in order to calculate GAD activity. A tissue homogenate was incubated with L-glutamate in sodium phosphate buffer at pH 6.4 and pyridoxal-5-phosphate. The limit of detection of the procedure was 10^{-7} mol GABA. Later MacDonnel and Greengard [48] compared Lowe's method [54] with that of a $^{14}CO_2$ technique. When Triton X-100 was utilized in an air atmosphere both procedures gave similar results of all tissues analyzed. Triton X-100 increased the fluorometric assay activity but a decrease in the isotopic assay activity was recorded in all cases. The detergent was believed to activate GAD and concurrently inhibit non-GAD CO_2 release from glutamate. The authors of this paper suggested that it was more reliable to quantitate GAD activity via GABA formation than CO_2 evolution since Triton X-100 may not completely inhibit non-GAD dependent decarboxylation of glutamate in all tissue homogenates. Holdiness et al. [43] reported improvements in the method of Lowe et al. [54]. In this procedure an improved sample collection procedure was described and the reaction of GABA with ninhydrin for fluorometric analysis had a detection limit of $0.20 \cdot 10^{-6}$ mol GABA. Activities reported in sub-regions of rat brain were 7.91 ± 1.47 (GP), 6.87 ± 2.07 (EP), 3.83 ± 0.69 (VM), 13.80 ± 2.14 (SN_m) and 8.23 ± 2.26 (SN_l) μg GABA per h per mg protein. Protein was measured by the method of Lowery et al. [55] and the anatomical tissue punch placement can be observed in Fig. 3 (atlas of Pellegrino and Cushman [56]).

3.3. Immunochemical assays

Immunochemical methods provide a powerful means for quantitation of the enzyme's concentration and anatomical location rather than its activity as previously discussed. The techniques use radioimmunoassay, microcomplement fixation and enzyme-labeled immunoassay procedures. These methods employ the making of a specific antibody against GAD [57]. Red blood cells are sensi-

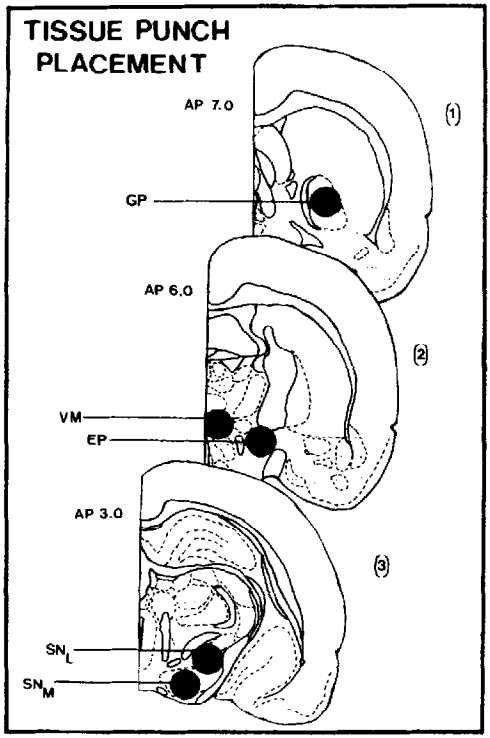

Fig. 3. Tissue punch placement. The first punch was from a 1-mm thick slice containing the globus pallidus (GP). The second slice contains the entopenduncular nucleus (EP) and ventromedial thalamus (VM). The third slice contains the substantia nigra medial (SN_M) and lateral (SN_L) punches. The numbers on the left refer to the anterior—posterior axis coordinates in the brain atlas of Pellegrino and Cushman [56]. Punch diameter, 1.30 mm.

tized with an antigen and reacted with anti-GAD immunoglobins. The amount of complement fixed after the antibody is added is proportional to the amount of antigen. This microcomplement fixation test can detect nanogram levels of GAD [58]. Radioimmunoassay and enzyme immunoassay procedures involve incubation of known quantities of the labeled enzyme antigen with unlabeled antigen. Binding is established by a standard curve and the concentration of the unknown antigen is determined. Also, peroxidase-labeled antibody is utilized for visualization of GAD at the light and electron microscopic levels in brain tissue [59—61].

4. CHROMATOGRAPHIC PROCEDURES

All chromatographic methods utilize the formation of GABA from L-GA as a means for GAD activity measurement. The product (GABA) is chromatographically separated from the homogenate incubation mixture before analysis. If radiolabeled GABA is formed, following separation from its radiolabeled substrate, it can be generally analyzed without further purification of the sample. However, if non-radioactive methods are employed a sample of tissue homogenate should be analyzed for GABA content prior to incubation. Following incubation, the original content of GABA present in tissues is subtracted from

that of the incubation mixture and a more precise measurement of GAD activity is acquired.

The chromatographic procedures to be described are finding wide utility in many laboratories. These methods offer similar sensitivity and generally better specificity for estimation of GAD activity in various biological media.

4.1. Paper and thin-layer chromatography

In 1959, Wilson et al. [62] reported what seems to be the first paper chromatographic procedure for separation of GABA. By this method [^{14}C]GA was incubated with a suspension of lyophilized cells of *Clostridium welchii*. The [^{14}C]GABA formed was separated by paper chromatography and identified via ninhydrin reaction and R_F values. GAD activity was not estimated; instead, the [^{14}C]GABA generated was utilized for further studies in rats and determined to be further metabolized to succinate in vivo. Kravitz [63] in 1962 incubated L-[3,4-$^{14}C_2$]GA and L-[U-^{14}C]GA with central ganglia and peripheral nerve bundles of lobsters. These incubations were lyophilized and separated by paper chromatography. The author observed with L-[U-^{14}C]GA that $^{14}CO_2$ and not radioactive GABA was formed; however, using L-[3,4-$^{14}C_2$]GA as substrate [^{14}C]GABA and not radioactive CO_2 was produced. Also noted in this experimentation was that GABA caused a reduction in the release of transmitters from excitatory nerve terminals at crayfish neuromuscular junctions. Prabhakaran and Braganca [64] published results indicating the presence of GAD activity from leprosy skin lesions. *Mycobacterium leprae* were separated from the skin nodules and incubated with and without L-GA. The preparations were able to decarboxylate GA and GABA was separated and identified via paper chromatography using ninhydrin for visible detection. DL-[3-$^{3}H_1$]GA in addition to DL-[U-^{14}C]GA have been used as substrates for enzymatic analysis [65]. In this case either substrate was incubated with lobster nerve cord and the reaction stopped by pipetting the mixture onto a Dowex-1 acetate chloride column. The GA was absorbed while GABA and other neutral amino acids passed through the column. The ^{14}C- or ^{3}H-radiolabeled GABA was then separated and identified by ascending paper chromatography. A Packard Strip Scanner was used to scan the radioactive chromatograms with nanogram levels of detection.

High-voltage paper electrophoretic procedures have been devised for GAD activity assays [66, 67]. As mentioned previously, Wu et al. [33] compared five different techniques of GAD analysis in different tissues one of which was electrophoretic. By this method the GABA concentration was determined by the ratio of total area of the electrophoresis to the area of the GABA peak. This method of analysis of GAD activity was found to be in agreement with the other procedures used except for the $^{14}CO_2$ method when tested in non-neural tissues.

In 1967 Homola and Dekker [68] observed the α-decarboxylation of a number of radiolabeled analogues of GA. The incubation mixtures were separated by two-dimensional TLC and the products quantitated radiometrically. Using this technique of separation and identification it was noted that the threo isomer of γ-hydroxy-L-glutamate could serve as a substrate for GAD with approximately 20% of the activity as that of L-glutamate. Wilson et al. [28] compared

the evolution of $^{14}CO_2$ from L-[U-^{14}C]GA with that of γ-amino[U-^{14}C]butyric acid formation in glial cell cultures. Using two-dimensional TLC for separation of radiolabeled GABA they observed up to 10% difference between the $^{14}CO_2$ evolution method as compared with [^{14}C]GABA. It was concluded that the increased $^{14}CO_2$ was being formed via alternative pathways other than that of GAD in these cell cultures. Miller and Martin [30] demonstrated by TLC that commercial L-[^{14}C]GA contained an impurity that produced excess CO_2. Aminooxyacetate, an inhibitor of GAD, was found to clearly stimulate $^{14}CO_2$ production from unpurified GA as when compared with production of [^{14}C]GABA from purified [^{14}C]GA in an incubation mixture. By this procedure, following incubation with purified [^{14}C]GA, the reaction products were separated by passing the contents through an AG1-X2 resin to retain GA and allow [^{14}C]-GABA to pass. The radiolabeled GABA was then further separated and purified on cellulose TLC plates and the spots quantitated by audioradiography. Wood et al. [69] also used cellulose thin-layer sheets for separation of GABA from mouse brain homogenates utilizing prepurified [^{14}C]GA as substrate. The solvent system consisted of chloroform—methanol—ammonia (2:2:1) and the chromatographic procedure required 35 min for development via a one-dimensional separation. The samples were analyzed with a scintillation counter and recovery was found to be 97% following deproteinization. Sellstrom et al. [70] and Prostenik et al. [71] both applied one-dimensional TLC for determination of GAD activity in brain homogenates. Radiolabeled GABA formed from the incubation mixture was identified by ninhydrin reaction and quantitated via scintillation counting in the former method [70]. The latter method of Prostenik et al. [71] used densitometry for quantitation of GABA. Pahuja and Reid [72] have published the latest known ion-exchange TLC method for analysis of GAD activity in bovine sub-retinal intercellular fluid and retina. The substrate (5 m*M*) of L-GA was incubated in the above mentioned homogenates (50 μl) with 50 m*M* potassium phosphate, pH 6.8, 1 m*M* EDTA, and 0.5 m*M* pyridoxal phosphate. The enzymatic reaction was stopped by immersion in ice water and 5 μl of the incubation mixture spotted on Ionex SB-Ac plates. Ascending TLC was carried out using water—ethyl acetate (92:8) and following chromatography the strips were cut out and analyzed by ninhydrin reaction. Separation of GABA (R_F 0.95) from glutamic acid (R_F 0.06) and other reaction products was obtained in 30 min. Fig. 4 presents the complete separation of radiolabeled GABA formed from the use of L-[3,4-$^3H_1$]glutamic acid as substrate when incubated with crude homogenates. Under the same conditions as described, incubation of the homogenate and radiolabeled GA without the enzyme for 2 h resulted in absolutely no formation of GABA by other enzymatic or non-enzymatic means. This present method was able to detect GAD activity in 1 μg of crude extracts when tritiated labeled GA (2000 cpm/nmol) was used and separation of the GA from GABA was not affected by changes in ionic strength or pH of the reaction mixture [72].

GAD activity has also been determined by the measurement of dansylated GABA. The reaction product from the incubation mixture was reacted with 5-dimethylamino-1-naphthalene sulfonyl chloride (dansyl chloride, Dns-Cl) via the procedure of Roberts et al. [73]. The samples were then separated on polyamide TLC plates and quantitated spectrophotometrically. Radiolabeled

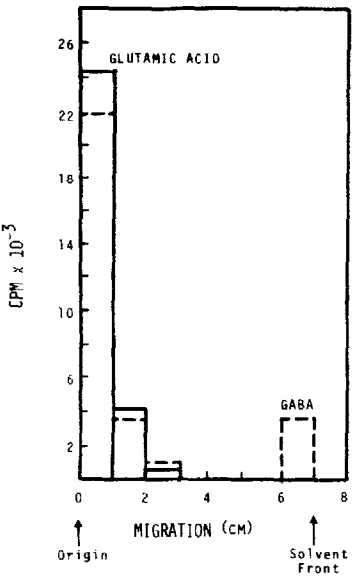

Fig. 4. Separation of [^{3}H]GABA (– – –) from [^{3}H]glutamic acid. After development, the strip was dried, cut into 1-cm pieces and radioactivity counted. The assay mixture was incubated with 180 μg of crude enzyme preparation at 37°C for 60 min. The control (—) contained the assay mixture mixed with enzyme at 37°C followed immediately by cooling at 0°C. The composition of the assay mixture has been described in the text. (Reproduced with permission from ref. 72.)

GA was also tested by this procedure and the [^{14}C]GABA formed was chromatographed under the same conditions as a test for the specificity of the analytical procedure. Morum and Wasterlain [74] reported the comparison of TLC separated dansylated [^{14}C]GABA with $^{14}CO_2$ production in tissue homogenates. Again the GAD values calculated by the CO_2 method were higher than the GABA method which lead the authors to suggest that the lack of stoichiometric agreement was probably due to alternative pathways and impurities in the substrate preparations. Osborne [75] reported the use of [^{14}C]dansyl chloride for derivatization of GABA from tissue homogenates. The fluorescent product was readily isolated by polyamide one-dimensional TLC and quantitated radiometrically. Later Osborne [76] introduced [^{3}H]dansyl chloride and reported detection limits of 1 pmol of GABA in nervous tissue. Strang [77] isolated GABA as the Dns-γ-butyralactone derivative with silica gel H. The solvent system used for chromatographic development was 18% ethanol in chloroform. The samples were analyzed radiometrically by $^{14}CO_2$ evolution and fluorescence and both gave comparable agreement.

4.2. Amino acid analyzers and ion-exchange chromatography

Wu et al. [34] demonstrated that L-[U-^{14}C]aspartic acid possessed about 3—5% of the activity found with L-glutamate as substrate in mouse brain extracts. The evidence came directly from α-decarboxylation and identification of the reaction products β-alanine and CO_2. These products were shown not to be due to impurity contamination in the L-glutamate. The reaction products were

identified with an amino acid analyzer on a cation-exchange resin and derivatized with ninhydrin for detection. Others had previously reported that bacterial GAD could decarboxylate α-methyl-DL-glutamic acid and L-glutamine in addition to L-glutamate [78]. Landcaster et al. [79, 80], Kravitz and Potter [81] and Moore and Stein [82] have described the analysis of GAD activity via amino acid analyzers. In the case of Landcaster et al. [79, 80] [^{14}C]GA was incubated with human kidney homogenates and the [^{14}C]GABA produced was separated from the substrate and homogenation mixture and analyzed by elution on an ion-exchange resin. The products were quantitated via scintillation counting. It was noted in these papers that renal GABA may play a role in acid—base metabolism of the kidneys.

Various ion-exchange methods have been used for GAD activity analysis [65, 81, 83, 84]. One procedure previously mentioned was that of Miller and Martin [30] in which a cation-exchange resin was used for partial purification of an incubation mixture prior to final analysis of GABA production via TLC and audioradiography. Chude and Wu [31] prepurified L-glutamate (radiolabeled) via ion-exchange chromatography prior to incubation. The substrate was incubated with GAD and the mixture separated by a Bio Rad AG1X column. The incubation mixture consisted of 0.1 ml of 0.1 *M* potassium phosphate, pH 7.2 with 0.2 m*M* pyridoxal phosphate, 1 ml 0.05 *M* potassium phosphate, pH 6.5 and 1 m*M* 2-aminoethylisothiouronium bromide, 0.2 m*M* pyridoxal phosphate and 0.2 ml 0.5 *N* sulfuric acid. The [^{14}C]GABA was completely eluted and GA was retained on the column by suction filtration. Radiolabeled GABA was measured by scintillation counting. This method offered advantages of rapid analysis and was more direct since CO_2 was not involved for analysis.

4.3. High-performance liquid chromatography

High-performance liquid chromatography (HPLC) methods for GABA and GAD activity analysis have become available only in recent years. These methods possess low detection limits and increased specificity for product measurement. Ion-exchange HPLC coupled with post-column derivatization and fluorescence detection has been applied for GABA analysis (GAD activity was not measured) by various investigators [85—89]. The limits of detection for these methods range from 1—50 pmol. Griesmann et al. [90] devised a fluorometric HPLC method for detection of dansylated GABA in rabbit cerebellum. This procedure involves pre-column derivatization with dansyl chloride and separation was effected with a reversed-phase column. The limit of detection was 100 pmol and this assay was compared with established TLC [91] and amino acid analyzer methods [82] using radiolabeled GABA. The HPLC procedure differed by +1.0% and −5.5% when compared with the TLC and amino acid analyzer methods, respectively [80]. Pahuja et al. [92] have also applied reversed-phase column separation of dansylated GABA from retinal cells. In this particular case GAD activity was measured via use of [^{3}H]GA as substrate. Following incubation of the homogenate with the radiolabeled substrate the products were derivatized in reaction vessels and lyophilized before HPLC injection. Ultraviolet (UV) absorption at 206 nm was used for detection (Fig. 5) and fractions were collected and analyzed via scintillation counting (Fig. 6A

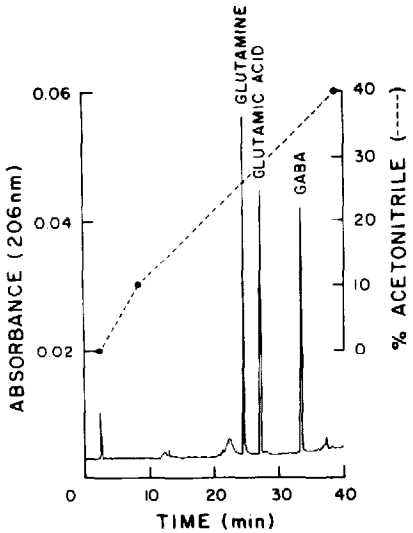

Fig. 5. Chromatogram showing the separation of 0.5 μg of dansylated glutamine, glutamic acid and GABA. The dashed line indicates the mobile phase gradient (% acetonitrile) used. (Reproduced with permission from ref. 92.)

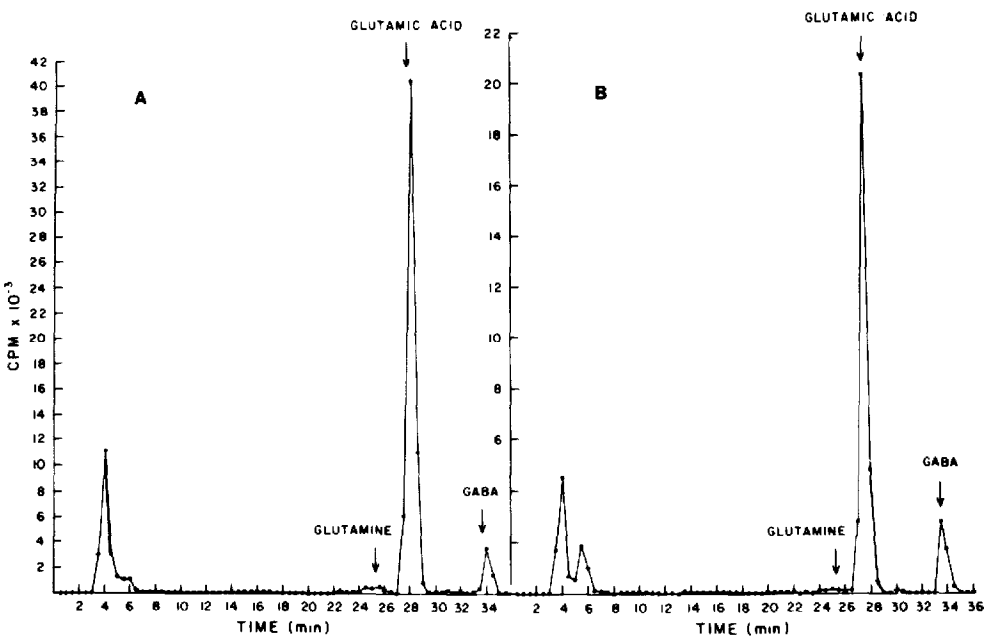

Fig. 6. Chromatograms of the reaction mixture of (A) crude brain extract under GAD assay conditions, 0.17 mg of crude enzyme protein plus phosphate buffer plus pyridoxal phosphate incubated for 1 h; (B) crude retinal extract under GAD assay conditions, 0.30 mg crude enzyme protein and phosphate buffer plus pyridoxal phosphate incubated for 2 h. If 0.5 m*M* pyridoxal phosphate was not added to either incubation mixture, GABA formation was not observed. (Reproduced with permission from ref. 92.)

and B). If pyridoxal phosphate was not added to the incubation mixture GABA formation was not observed. Gradient elution with acetonitrile was required for chromatographic separation of the derivatized products. As little as 100 ng of the product could be detected by this procedure using UV detection.

Recently electrochemical detection coupled with HPLC separation has been applied for GABA analysis [93]. GABA and the internal standard, 5-aminovaleric acid (5-AVA), were derivatized with 2,4,6-trinitrobenzenesulfonic acid and the components detected by electrochemical reduction with an annealed pyrolytic graphite electrode held at −0.8 V vs. saturated calomel electrode. Either a strong cation-exchange or a reversed-phase column were used for separation; limits of detection of GABA extracted from rat brain were 4 pmol. This method of analysis has yet to be applied for measurement of GAD activity in biological tissues; however, it should provide a very sensitive and specific technique for small sample size such as specific nuclei in brain tissues.

Post-column derivatization with *o*-phthalaldehyde using cation-exchange and reversed-phase columns have been applied to fluorometric GABA analysis in biological tissue [86, 94—97]. These methods were not used to measure GAD activity. Probably the first reported HPLC procedure used for GAD activity analysis was that of Meek [98]. Brain homogenates were incubated at 37°C for 30 min with GA as substrate and following denaturization of the enzyme via precipitation of the supernate was separated on a strong cation-exchange column before analysis. In this particular case an internal standard was not used for quantitation. Holdiness [99] reported a fluorometric procedure for analysis of GAD activity in sub-regions of rat brain using a cation-exchange column. The sample collection procedure has been previously described [43] and the

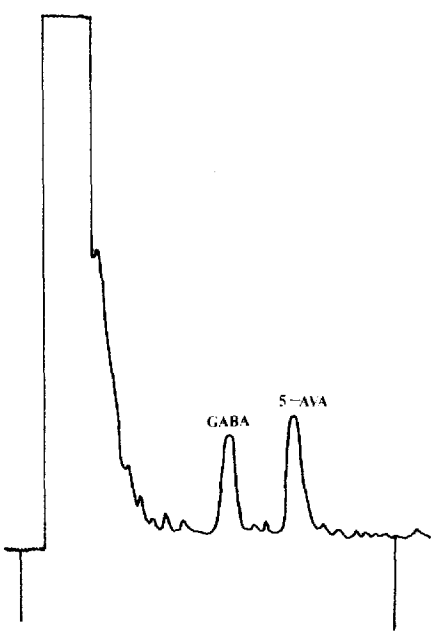

Fig. 7. HPLC chromatogram of GABA and 5-aminovaleric acid (5-AVA internal standard) extracted from rat brain tissue. The retention times of GABA and internal standard are 4.70 and 6.86 min, respectively.

tissue punch placement can be seen in Fig. 3. From each 1-mm thick brain tissue slice, two tissue punches were taken from symmetrical locations in the left and right hemispheres. Each set of punches was immediately transferred to an eppendorf tube containing 100 μl of internal standard (5-AVA) and homogenized for 10 sec under low-power sonication. Following sonication, 50 μl of this solution was transferred to an identical tube containing 20 μl of 3% trichloroacetic acid (TCA). This second tube served as a blank and its concentration of GABA was subtracted from the original sample. The substrate—buffer was prepared as previously described [43] and 50 μl of this solution was added to both the sample and blank tubes before they were incubated for 2 h at 38°C. The enzyme was inactivated by addition of 20 μl of 3% TCA to the original sample tube and all tubes were centrifuged at 950 *g* for 20 min. Injections of 40 μl of the supernate were made into the chromatograph. The precipitate was analyzed for protein as described by Lowery et al. [55] and GAD activity was reported in μg GABA per h per mg protein. Fig. 7 shows a typical chromatogram of GABA and internal standard isolated from rat brain. Positive identification was achieved by peak superimposition, i.e., by addition of GABA and 5-AVA standard to the extracts and observing increased peak height at the corresponding retention times. The lower limit of detection (signal-to-noise ratio = 2:1) of this procedure was 11 ng GABA. The average GAD activities (± S.D.) found by this method in each brain region (n = 5) were 7.81 ± 1.08 (GP), 6.73 ± 1.58 (EP), 3.75 ± 0.71 (VM), 13.70 ± 1.75 (SN_M) and 8.17 ± 1.68 (SN_L).

4.4. Gas chromatography—mass spectrometry

A number of gas chromatographic (GC) procedures have been developed for analysis of GABA content in biological media without necessarily being applied for GAD activity measurements. Many of these procedures have provided very sensitive and specific means of detection and quantitation. Flame ionization detection (FID) methods include the procedures of Shimada et al. [100] who made the N-trifluoroacetyl-*n*-butyl ester of GABA and the N-trifluoroacetyl-methyl esterification method for GABA by Carlyle [101]. Also direct injection of GABA into the gas chromatograph following extraction has been utilized [102]. The lactam product of GABA, 2-pyrrolidone, resulting from dehydration of GABA from a Carbowax column, was identified by mass spectrometry (MS). Electron-capture gas chromatography (EC—GC) has been employed for greater sensitivity and specificity of analyses. Wilk and Orlowski [103] and Pearson and Sharman [104] have demonstrated the use of combinations of 2,2,3,3,3-pentafluoropropionyl-2,2,3,3,3-pentafluoropropyl and 1,1,1,3,3,3-hexafluoroisopropyl-trifluoroacetyl derivatives, respectively, for GABA derivatization. The latter reagent formed easily at room temperature and possessed excellent EC—GC properties. The limit of detection was 10 pmol GABA in rat brain tissue. The extraction procedure of Pearson and Sharman [104] was later improved by Schmid and Karobath [105]. The same derivatization agents were used for GC—MS identification of the product and its internal standard (5-AVA). Both EC—GC and GC—MS procedures gave similar results for GABA analyzed from brain tissue. Tago et al. [35] applied an EC—GC method to the analysis of GABA by enzymatic activity in brain tissue. The derivatization

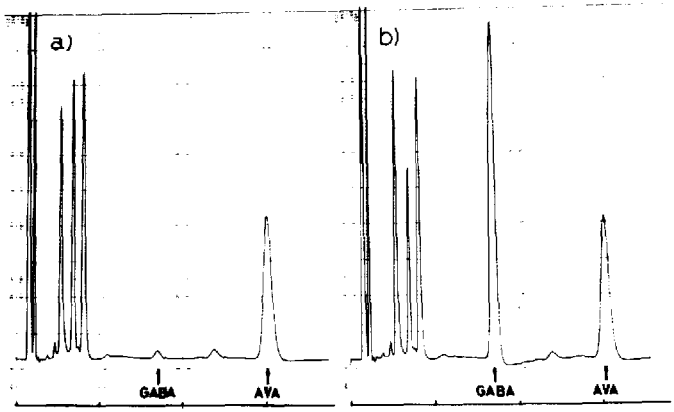

Fig. 8. Gas chromatograms of the acylated GABA and 5-aminovaleric acid (AVA). GABA formed from 4-aminobutyraldehyde dehydrogenase was acylated with trifluoroacetic anhydride and hexafluoroisopropanol, together with the internal standard. (a) represents the control and (b) represents the formation of GABA following a 10-min incubation period. (Reproduced with permission from ref. 35.)

agents were trifluoroacetic anhydride (TFAA) and hexafluoroisopropanol (HFIP) with 5-AVA as internal standard. Fig. 8 presents a typical chromatogram from their data. This method of analysis has been utilized for demonstration of evidence of synthesis of GABA from 4-aminobutyraldehyde dehydrogenase [35].

A number of GC—MS techniques have also been applied for GABA measurement in brain tissue [102, 105—112]. Cattabeni et al. [113] and Holdiness et al. [106] developed mass fragmentographic methods utilizing hexamethyldisilane, trimethylchlorosilane, and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) as the derivatizing agents forming trimethylsilyl (TMS) derivatives of the amine and carboxylic acid moieties of this molecule. The internal standard was 5-AVA and separation was performed on a 3% OV-17 column (Gas-Chrom Q), 100—120 mesh at 140°C. These two GC—MS procedures detected GABA in rat brain tissue with nanogram sensitivity. Employing the technique of selective ion monitoring (SIM) ions *m/e* 174 and 304 (GABA) and *m/e* 174 and 318 (5-AVA) were utilized for quantitation in brain tissue.

Huizinga et al. [114] and Bertilsson and Costa [115] both utilized GC—MS methods for GABA analysis in cerebral spinal fluid and brain tissue, respectively. Both procedures used a deuterated internal standard [$^2H_2$]GABA and pentafluoroproprionic anhydride (PFPA) and HFIP as derivatizing agents. The ions selected for quantitation by Huizinga et al. [114] were *m/e* 204 (GABA) and *m/e* 206 (deuterated internal standard). Monitoring ions *m/e* 232 (GABA) and *m/e* 234 (deuterated internal standard) gave essentially the same standard curve and quantitative results. Ions *m/e* 204 (GABA) and *m/e* 206 (internal standard) were utilized for SIM by Bertilsson and Costa [115] for their mass fragmentographic analysis.

To date, only two mass fragmentographic techniques have been developed for measurement of GAD activity in biological tissues. Cattabeni et al. [116] described a procedure in which rat cerebellum tissue homogenate was incubated with [$^2H_5$]GA as substrate and measured the formation of [$^2H_5$]GABA. The

internal standard 5-AVA was also added to the homogenate for comparison of measurement and ions *m/e* 304 (GABA), *m/e* 309 ([$^2H_5$]GABA) and *m/e* 318 (5-AVA) were monitored for quantitation. The derivatization procedure and chromatographic conditions have been previously described [113]. The standard curves for the two different internal standards revealed essentially the same data for GABA content in rat cerebellum. However, it was not determined whether any isotopic discrimination occurred with the enzyme and the deuterated substrate.

Holdiness et al. [117, 118] have described the latest known mass fragmentographic method of analysis of GAD activity in sub-regions of rat brain tissue. The internal standard was [$^2H_2$]GABA and the derivatization procedure utilized Sylon HTP [hexamethylchlorosilane—trimethylchlorosilane—pyridine (3:1:9)] and BSTFA for 90 min at room temperature. Separation was performed on a 3% OV-17 column (under the conditions described [106]) at 160°C. Ions *m/e* 304.1 (GABA) and *m/e* 306.2 ([$^2H_2$]GABA) were selected for quantitation. The limit of detection for this particular GC—MS method was 15 ng GABA per mg tissue. The sample collection procedure and substrate—buffer preparations have been previously described [43, 99]. Following homogenization the sample was divided into two fractions; one was inactivated with TCA and served as blank while the other portion was incubated under standard conditions as previously stated for the fluorometric HPLC method [99]. The concentration of GABA from the blank was subtracted from that of the incubated mixture. Following enzyme inactivation with TCA and centrifugation an 80-μl aliquot

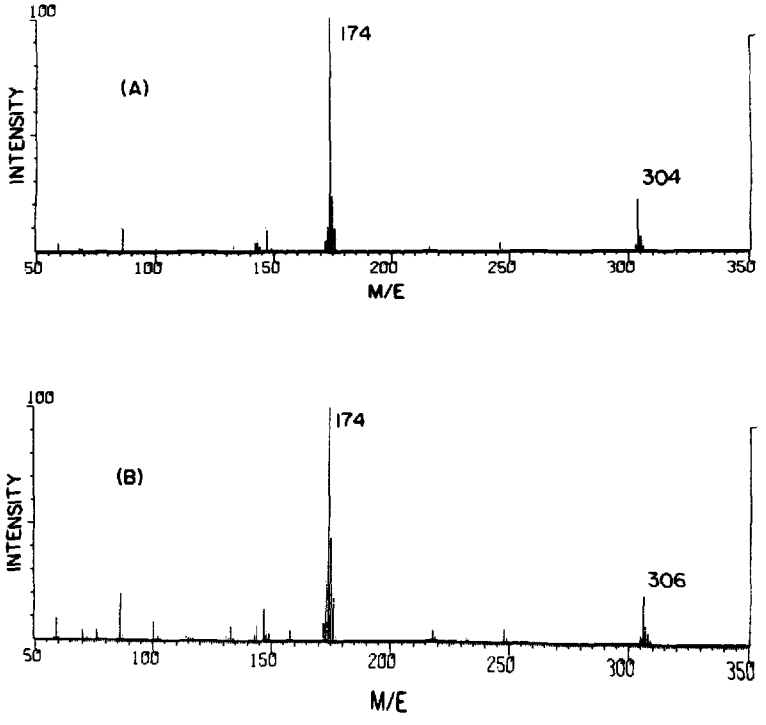

Fig. 9. Mass spectra of the trimethylsilyl derivatives of (A) GABA and (B) [$^2H_2$]GABA at 70 eV.

of the sample was transferred to a small 1.5-ml eppendorf tube and evaporated to dryness under reduced pressure. The samples were then derivatized as stated above and analyzed by GC—MS.

A mass spectrum of the TMS derivative and deuterium labeled derivative is presented in Fig. 9. The base peak in both spectra is *m/e* 174. The ions monitored $(M - 15)^+$ by electron impact at 70 eV were *m/e* 304.1 (GABA) and *m/e* 306.2 ($[^2H_2]$GABA). The relative abundancies of *m/e* 304.1 and *m/e* 306.2 are 22% and 20%, respectively. From mass spectra analysis, $[^2H_2]$GABA gave a small contribution to *m/e* 304.1 of 0.22% relative abundance while GABA has an ion of 12.83% relative abundance at *m/e* 306.2. The signal intensities of the above mentioned ions are 2.5 times stronger at 70 eV than at 30 eV.

To test whether greater sensitivity could be achieved, spectra of pure standards of each compound were obtained using chemical ionization with methane as reagent gas. In both samples the base peak was *m/e* 174. The $(MH)^+$ ions of GABA (*m/e* 320) and $[^2H_2]$GABA (*m/e* 322) were both present at 7% relative abundance. At 70 eV the $(M - 15)^+$ relative abundancies of *m/e* 304.1 (GABA) and *m/e* 306.2 ($[^2H_2]$GABA) were 75% and 73%, respectively. It was found that the chemical ionization signal intensities of these two ions were 4.25 times less than the signal intensities at 70 eV by electron impact. Therefore, electron impact was used in all GABA measurements.

Fig. 10 presents a mass fragmentogram of one of the tissue samples. The chromatographic retention time of the endogenous compound was identical to that of its deuterated analogue. The average GAD activity values (± S.D.) found by this method in each brain region ($n = 5$) were 7.75 ± 0.93 (GP), 6.70 ± 1.02 (EP), 3.68 ± 0.70 (VM), 13.66 ± 1.06 (SN_M) and 8.12 ± 0.95 (SN_L). These

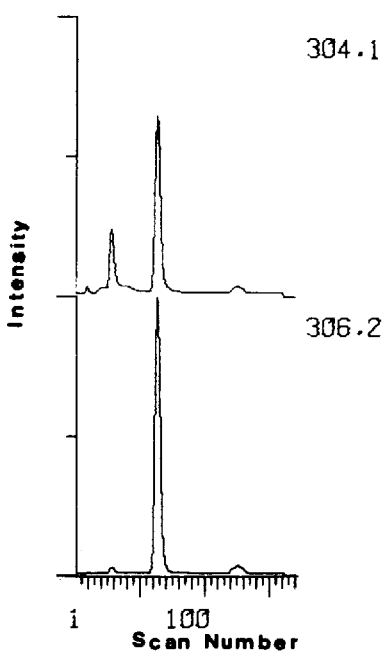

Fig. 10. Mass fragmentogram of *m/e* 304.1 of GABA and *m/e* 306.2 of $[^2H_2]$GABA from a tissue sample.

brain punch samples weighing approximately 2.50 mg each were taken from the same locations as presented in Fig. 3. By measurement of a characteristic ion of GABA and comparison of its retention time to that of a deuterated internal standard, this assay method represents a very specific, selective and sensitive method for GAD activity measurement.

5. CONCLUSION

Numerous chromatographic and non-chromatographic methods for analysis of GAD activity in biological tissues have been discussed in this manuscript. Methods are generally devised or selected for use according to the investigator's expertise and possible prior experience with either radioactive materials, fluorometric or chromatographic techniques and the type of instrumentation that is available in their laboratory. Therefore, of the procedures available major consideration must be given to the reliability, specificity, selectivity and reproducibility among the different instrumental techniques and their comparison with different tissues. As has been described in this paper differences of up to 10% have been noted between $^{14}CO_2$ and [^{14}C]GABA methods utilized for GAD analysis due to alternate pathways of production of carbon dioxide [28, 46], impurities in the enzyme and substrate preparations, and reaction vessel atmospheres [29—31, 33]. MacDonnel and Greengard [48] compared a $^{14}CO_2$ production method with that of a fluorometric procedure. They demonstrated that the addition of Triton X-100 (utilized in an air atmosphere) modified the $^{14}CO_2$ method in such a way that it inhibited non-GAD CO_2 release from glutamate. Following modifications both methods produced similar GAD activities in both neural and non-neural tissues. Wu et al. [33] measured GAD activity in brain and non-neural tissues by five different analytical techniques ($^{14}CO_2$, column separation, electrophoretic separation, a filtration method and by amino acid analysis). The last four procedures involved analysis of GABA production as opposed to carbon dioxide formation. Purified L-[U-^{14}C]GA was utilized as substrate. All five methods gave similar GAD activities in brain tissue; however, for tissues as heart, kidney and liver, the $^{14}CO_2$ assay technique revealed GAD activities approximately three to four times that of the GABA formation methods. This suggested that the CO_2 method does not give valid quantitation of GAD activity in crude non-neural tissue preparations [12, 33].

This author has developed three analytical instrumental techniques (fluorometric [43], HPLC [99] and GC—MS [117, 118]) for analysis of GAD activity in rat brain tissue. The same collection procedure, strain of rats and tissue punch locations (see Fig. 3) were utilized for all three methods. In Fig. 11 is presented a bar graph of the GAD activity found at each location by each analytical method. As can be seen by the data each analytical technique gave approximately the same GAD activity in each sub-region of rat brain; therefore, demonstrating the reliability, selectivity and reproducibility of each different method. The slight decrease in average GAD activity at each sub-region from fluorometric to HPLC to GC—MS is attributed to the specificity of the technique used. The GC—MS method is considered to be the most specific since a characteristic ion fragment is monitored for analysis.

The GAD activities found by this author in the above three listed procedures

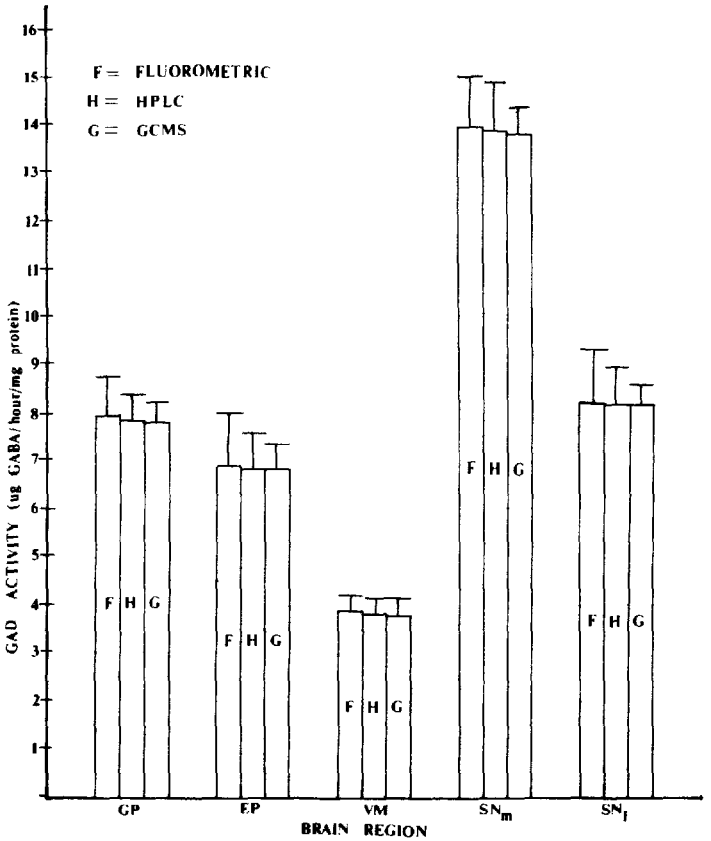

Fig. 11. Bar graph of GAD activity versus sub-region in rat brain for three different analytical techniques. (F) is a fluorometric method [43], (H) is an HPLC method [99], and (G) is a GC—MS procedure [117, 118].

TABLE 2

A COMPARISON OF RATIOS OF GAD ACTIVITY IN SUB-REGIONS OF RAT BRAIN BY VARIOUS ANALYTICAL METHODS

Analytical technique	GP/EP sub-region	GP/VM sub-region	Reference
Fluorometric	1.15	2.06	Holdiness et al. [43]
Fluorometric HPLC	1.16	2.08	Holdiness [99]
GC—MS	1.16	2.11	Holdiness et al. [117]
$^{14}CO_2$	1.80	—	Nagy et al. [14]
$^{14}CO_2$	1.93	—	Walaas and Fonnum [8]
$^{14}CO_2$	—	2.03	Tappaz et al. [119]

are in agreement with those found by Nagy et al. [14], Walaas and Fonnum [8] and Tappaza et al. [119] in the GP, EP and SN. A comparison of regional GAD activities has been made with these data and that in the previously cited literature and is presented in Table 2. The author choose what he considered the best brain tissue comparisons (regardless of analytical technique) with respect

to anatomical location. Due to the different ways activity values are reported (i.e., per mg tissue, mg protein dry weight, wet weight) ratios of activities between regions have been used for comparison. Although a direct comparison of these ratios is not ideal, the ratios should be relatively consistent from method to method. As can be seen by the data in Table 2 there are comparable results reported by this author and that of the previously cited literature. Possible discrepancies among these activity values could be due to extraneous $^{14}CO_2$ production from alternative pathways, impurities in substrate mixtures, variations in anterior—posterior axis location in brain regions, differences in protein content among sub-regions, comparison of whole dissected regions as opposed to micropunches and differences in strains of rats tested.

As previously mentioned there seems to exist evidence for GABA production from an alternative pathway of 4-aminobutyraldehyde dehydrogenase (4-ABALD) in rat brain [35, 120, 121]. Also a synthetic pathway for GABA formation from putrescine has been proposed by Seiler et al. [122] in fish brain and Seiler and Al-Therib [123], Seiler et al. [124] and Sugahara et al. [125] in mammalian brain although it has not been fully clarified. It is possible that 4-ABALD may be involved in the conversion of putrescine to GABA in mammalian brain [35]. Although alternative routes of GABA formation may exist in neural tissue, their incubation conditions are different from those of the established conditions for GAD analysis; therefore, it seems unlikely that extraneous GABA would be produced to any significant extent by alternative pathways under these stated reaction conditions. This fact also seems to be verified by the method of Pahuja and Reid [72] who demonstrated that crude retinal incubation homogenates did not produce GABA without the presence of the GAD enzyme in the reaction vessel.

6. SUMMARY

A number of non-chromatographic and chromatographic methods for analysis of GAD activity in biological tissues have been described. The majority of these chromatographic methods utilize the analysis of GABA formed from incubation homogenates. Depending upon the analytical technique selected, limits of detection range from nanogram to picogram levels of GABA. Also discussed have been some of the commonly associated problems and their resolution with sample collection, postmortem changes and alternative pathways of CO_2 and GABA production which can lead to errors in accurate determination of GAD activity in biological samples.

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