A NEW MICROASSAY METHOD FOR L-GLUTAMIC ACID DECARBOXYLASE (GAD) ACTIVITY

Hiroshi KIMURA and Kinya KURIYAMA
Department of Pharmacology, Kyoto Prefectural University of Medicine, Kamikyo-ku, Kyoto 602, Japan
Accepted February 5, 1975

Abstract- A simple and sensitive microradiometrical assay method for L-glutamic acid decarboxylase (GAD: EC 4.1.1.15) has been designed. Cerebral tissue was frozen and sectioned at 300 μ thickness, after which the slice was further dissected into rectangular blocks (750×750 μ). The GAD activity was assayed microradiometrically. The incubation vessel held a volume of approximately 500 μl and the incubation mixture was 90 μl. The 14CO2 evolved from L-[1-14C]-glutamic acid was absorbed with a filter paper immersed in 100 μl of hyamine base. GAD activity in neuronal tissues containing less than 5-10 μg of protein was easily and accurately detected by this method. The assay was found to be linear at 5-500 μg of protein concentration in various CNS preparations. Utilizing this microradiometric assay method, it was found that in the rat hypothalamus, the distribution pattern of GAD activity was essentially the same as that of GABA.

Gamma-aminobutyric acid (GABA) is considered to be an inhibitory neurotransmitter in the mammalian central nervous system (CNS) (1, 2). For the identification of GABA containing neurons in the mammalian CNS, specific and sensitive methods for the measurement of the GABA content as well as activities of GABA-transaminase (GABA-T: EC 2.6.1.19) and L-glutamic acid decarboxylase (GAD: EC 4.1.1.15) are required. Although regional or cellular distributions of GABA and GABA-T are measurable respectively by the method of enzymatic cycling (3) for the measurement of GABA (4, 5) and the histochemical method for GABA-T(6), a suitable microassay method for GAD activity has not been reported. The level of GAD activity in neuronal tissues has been determined by measuring GABA formation fluorometrically (7), or by measuring CO2 evolved from L-glutamate manometrically (8) or radiometrically (9, 10). The most sensitive method is the radiometric procedure which is performed by using L-[^14C]-labelled glutamic acid as the substrate and measuring the 14CO2 evolved. Albers and Brady (11), and Molinoff and Kravitz (12) have already reported a microradiometrical assay method for GAD. Their procedures, however, cannot be employed under anaerobic conditions, and are time consuming and tedious when a large number of small samples are being handled.

This paper describes a radiometric microassay method capable of measuring GAD activities in the tissue containing as little as 5-10 μg of protein. Regional distributions of GAD activity determined by this microassay technique are also presented.
MATERIALS AND METHODS

Male albino rats of Wistar strain weighing 130-150 g were decapitated and the brain rapidly frozen by liquid CO₂ expansion. The frozen cerebral tissue was sectioned with a cryostat at 300 μ thickness. The sections were slightly melted for easy mounting on the glass slide covered with thin paraffin layer and photographed immediately under a dissecting microscope to determine the stereotaxic coordinates according to the stereotaxic atlas of Pellegrino and Cushman (13). The same section was then refrozen in a cold box described by Aprison and Hancock (14), and was further cut rectangularly (750 × 750 μ) under a dissecting microscope which was attached to an ocular-miriometer for calibration. The location and volume of these blocks were determined photographically.

For the GABA assay, each block was transferred into 1 ml-fluorometer tube (conical type, 1 ml in a total volume) containing 75% ethanol. After evaporating ethanol in a dessicator under vacuum, the GABA content in these samples was assayed by the enzymatic procedure of Graham and Aprison (15). For the determination of GAD activity, the dissected tissue block was transferred into an ice-cold microhomogenizer and homogenized at 2°C in 50 μl of nitrogen-gassed distilled water containing 0.25% (v/v) of Triton X-100 to ensure maximal liberation of the enzyme. The homogenate was further stored on ice for 20 min to ensure osmotic liberation of the enzyme, meanwhile 5 μl portion of the homogenate was taken for the measurement of protein content by a slight modification of the procedure of Lowry et al. (16). Next 40 μl portion of the homogenate was transferred into a specially designed micro-incubation vessel (total vessel volume: approx. 500 μl, see Fig. 1) containing 50 μl aliquot of the incubation medium which consisted of 0.33 μCi of L-[1-14C]-glutamic acid (29 mCi/mM), 5 μmoles of carrier L-glutamic acid, 10 nmoles of pyridoxal phosphate, 100 nmoles of aminooethylisothiouronium bromide hydrobromide (AET) and 10 μmoles of potassium phosphate buffer at pH 6.4 (17). The incubation vessel was capped with a glass stopper containing a folded filter paper (15 × 40 mm) immersed in 0.1 ml of hydroxide of hyamine base for the absorption of 14CO₂ evolved from L-[1-14C]-glutamic acid (see Fig. 1). After gassing through the vessel with purified nitrogen gas from a side arm for 1 min, the side arm was capped with a rubber stopper and the glass stopper was turned to seal air tight. Incubations were carried out for 60 min at 37°C in a metabolic incubator. After terminating the reaction by the injection of 50 μl of 5N-H₂SO₄ with a tuberculin syringe by means of a needle through the
rubber stopper, the vessels were further incubated for an additional 90 min to achieve quantitative absorption of $^{14}$CO$_2$ by hyamine base (18). The content of glass stopper in an incubation vessel was transferred into a counting vial containing 12 ml of Toluene scintillation cocktail (dissolved 5.0 g PPO and 0.3 g POPOP in 1000 ml of toluene), whereas a 50 μl portion of incubation medium was pipetted into a counting vial containing 12 ml of Bray’s scintillation fluid mixture (19). The radioactivity in these vials was determined in a Packard 3379 liquid scintillation spectrometer equipped with automatic quenching correction which was utilized for all determinations reported here. In all experiments, blank vessel without tissue was incubated simultaneously and the minimal amount of $^{14}$CO$_2$ evaporated in the blank was subtracted from each experimental value.

RESULTS AND DISCUSSION

For the estimation of protein content in a small volume of sample (5 μl), the assay reagent described by Lowry et al. (16) was used with slight modification. In the reagent B (copper tartrate solution) 2.5 ml volume of 1N-NaOH was added to the mixture of 50 ml of 2% Na tartrate and 50 ml of 1% copper sulfate to stabilize the reagent (20). The volume of samples was decreased to the extent of 5 μl, and as little as 0.5 μg of protein could be measured accurately when conventional bovine albumin (Sigma, bovine albumin, fraction V powder) was used as working standards (Fig. 2-A). However, it must be noted that the blank values in protein measurement were fairly high due to the presence of Triton x-100. When employing a Shimazu (Model QV-50) spectrophotometer, the optical density at 750 nm for the blank, containing the same amount of Triton x-100 as tissue samples, was usually approx. 0.060.

Fig. 2.- (A) : A standard curve for protein assay by a modified method of Lowry et al. (16). The volume of samples used was 5 μl. 2.- (B) : Proportionality of GAD activity (expressed as $^{14}$CO$_2$ evolved) to protein content is shown. The experiments were employed in the phase of N-gas or air (see methods for details of the assay). Thirty to 50 μg of tissue protein was used. Each point represents the mean obtained from three separate determinations.
GAD activity expressed as $^{14}$CO$_2$ evolved was sufficiently proportional to protein content in various CNS preparations examined (Fig. 2-B). In addition, the GABA contents in the reaction mixture before and after incubation were measured fluorometrically and it was found that values for GAD activity measured by this microradiometric procedure and those obtained by direct measurement of GABA were essentially in parallel. Although the GAD activities measured under gas phases of nitrogen and air respectively were found to be linear at 20–500 μg of protein content, higher activity and more reproducible results were obtained in the nitrogen-gassed condition than those in air phase. Superiority of the anaerobic assay system in terms of reproducibility in the assay results was evident particularly in assays using low protein concentrations. The minimal amount of tissue protein required for this microradiometrical assay for GAD activity was less than 5–10 μg.

Effect of Triton x-100, which was added with a concentration of 0.25% (v/v) to nitrogen-gassed distilled water for the homogenization, on GAD activities is shown in Fig. 3. In the case of three different incubation mixtures, the enzyme activity was significantly increased by the addition of Triton x-100 to the tissue homogenates. These results indicate that the addition of Triton x-100, a non ionic detergent, to the assay system is necessary for achieving complete liberation of the enzyme from particles, as previously suggested (21). It was also noted that the enzyme activity in the presence of sulfhydryl compound, especially in the presence of AET, was higher than the control values. By using both AET and pyridoxal phosphate under nitrogen-gassed condition, a decrement of the enzyme activities was not found during the 60 min incubation (Fig. 3). Roberts and Simonsen (22) suggested that the enhancement of the activity by sulfhydryl compound is probably attributable to the protection of the sensitive sulfhydryl groups of the enzyme against oxidation by dissolved oxygen. Our data in Table I support this hypothesis. Aminooxyacetic
### Table 1. Effect of AET, AOAA and NaCl on GAD activity of cerebellar tissues of the rat

| Drugs added (mM) | GAD activity (μg GABA formed/mg protein/hr) |
|------------------|--------------------------------------------|
|                  | O$_3$-gassed                               | N$_2$-gassed                               |
| PLP (0.1)        | 6.60±0.42                                  | 26.33±0.72                                 |
| PLP (0.1), AET (1)| 15.04±0.07                                 | 31.68±2.17                                 |
| PLP (0.1), AET (1), AOAA (0.1) | 6.53±1.13                                  | 7.84±0.73                                  |
| PLP (0.1), AET (1), NaCl (100) | 12.92±0.09                                  | 28.57±1.46                                 |

Thirty to 50 μg of tissue protein was used in the enzymatic assays. Each value is the mean ± S.D. obtained from three separate determinations. Abbreviations used: AET, aminoethylisothiouronium bromide hydrobromide, AOAA, aminooxyacetic acid, PLP, pyridoxal phosphate.

### Table 2. Distribution of GAD activity in gross regions of the rat CNS

| Tissue                | GAD activity (μg GABA formed/mg protein/hr) |
|-----------------------|--------------------------------------------|
| Whole brain           | 13.9±2.5                                   |
| Cerebral cortex       | 20.3±2.1                                   |
| Midbrain              | 34.8±4.2                                   |
| Cerebellum            | 23.1±2.6                                   |
| Pons and Medulla      | 9.1±2.0                                    |

Protein contents in the tissue samples used for the measurement of the enzyme activity was approx. 10-30 μg. Each value represents the mean ± S.D. obtained from three separate determinations.

**Fig. 4.** Distribution of GABA and GAD activities in the rat hypothalamus. The section at 6.4 mm anterior from interaural line is shown (A 6.4) according to the stereotaxic atlas of Pellegrino and Cushman (13).

Abbreviations used: AM, amygdaloid nuclei complex; AHA, anterior hypothalamic area; ARH, arcuate nucleus of hypothalamus; CC, corpus callosum; CI, internal capsule; CPU, caudate nucleus; FX, fornix; GP, globus pallidus; LHA, lateral hypothalamic area; OT, optic tract; PVH, paraventricular nucleus of hypothalamus; SO, supraoptic nucleus of hypothalamus; VMH, ventromedial nucleus of hypothalamus.

The mean ± S.D. obtained from nine separate experiments is shown.

* Corresponded to the numbers represented in the figure on the left hand side.
acid (AOAA), a carbonyl trapping agent which is known as a potent inhibitor of GAD (22), strongly inhibited the enzyme activity in both O₂ or N₂ gassed conditions. It was also observed that GAD activity is inhibited by the addition of high concentrations of NaCl. This weak inhibition by NaCl probably can be attributed to the well known Cl⁻ inhibition on GAD activity (17). Effects of these two inhibitors on the enzyme activity are essentially in agreement with those reported by other investigators (22, 23). Table 2 illustrates the distribution of GAD activities measured by this microradiometrical method in gross regions of the CNS. The values in Table 2 are essentially in good agreement with those obtained by the conventional radiometric method (24, 25) or the fluorometric method (7).

A typical example of the application of this microradiometric method for determining the regional distribution of GAD activity in the hypothalamus is shown in Fig. 4. One side of various hypothalamic blocks was used for the determination of GAD activity, and blocks obtained from the other side were utilized for the measurement of GABA content. Although hypothalamic areas showing high concentrations of GABA and GAD activity did not coincide with the location of hypothalamic nuclei, distribution patterns of both GABA and GAD activity were essentially identical in this part of the brain. The highest GABA content and GAD activity were constantly observed in the lateral hypothalamic area. Considering the necessity of the measurement of GAD activity for identifying GABA containing neurons in the CNS, the sensitive microuassay method for the GAD activity reported herein will provide a useful tool for this purpose.

Acknowledgement—This study was supported in part by research grants (Nos. 744023, 977058 and 987015) from the Ministry of Education, Japan.

REFERENCES
1) ROBERTS, E. AND KURIYAMA, K.: Brain Res. 8, 1 (1968)
2) BAXTER, C.F.: Hand Book of Neurochemistry (Lajtha, A. ed.), Vol. 3, pp. 289-354, Plenum Press, New York and London (1970)
3) LOWRY, O.H., PASSONNEAU, J.V., SHULZ, D.W. AND ROCK, M.K.: J. biol. Chem., 236, 2746 (1961)
4) TACHIBANA, M. AND KURIYAMA, K.: Brain Res. 69, 370 (1974)
5) OTSUKA, M., OBATA, K., MIYATA, Y. AND TANAKA, Y.: J. Neurochem., 18, 287 (1971)
6) VAN GELDER, N.M.: J. Neurochem., 12, 231 (1965)
7) LOWE, I.P., ROBINS, E. AND EYERMAN, G.S.: J. Neurochem., 3, 8 (1958)
8) ROBERTS, E. AND FRANKEL, M.: J. Biol. Chem., 188, 789 (1951)
9) ROBERTS, E. AND SIMONSEN, D.G.: Biochem. Pharmacol., 12, 113 (1963)
10) SISKEN, B., SANO, K. AND ROBERTS, E.: J. Biol. Chem., 236, 503 (1961)
11) ALBERS, R.W. AND BRADY, R.O.: J. Biol. Chem., 234, 926 (1959)
12) MOLINOFF, P.B. AND KRAVITZ, E.A.: J. Neurochem., 15, 391 (1968)
13) PELLEGRINO, L.I. AND CUSHMAN, A.J.: A Stereotaxic Atlas of the Rat Brain. Appleton-Century-Crofts, New York (1967)
14) APRISON, M.H. AND HANCOCK, C.J.A.: Experiments in Physiology and Biochemistry, Edited by KERKUT, G.A., Vol. 3, p. 39, Academic Press, New York (1970)
15) GRAHAM, L.T. AND APRISON, M.H.: Analyt. Biochem., 15, 487 (1966)
16) LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. AND RANDALL, R.J.: J. Biol. Chem., 193, 265 (1951)
17) SUSZ, J.P., HABER, B. AND ROBERTS, E.: Biochemistry, N.Y. 5, 2870 (1966)
18) PASSMANN, J.M., RADIN, N.S. AND COOPER, J.A.D.: Analyt. Chem., 28, 484 (1956)
19) Bray, G.A.: *Analyt. Biochem.*, 1, 279 (1960)
20) Hess, H.H. and Lewin, E.: *J. Neurochem.*, 12, 205 (1965)
21) Van Kempen, G.M.J., Van den Berg, G.J., Van del Helm, H.J. and Veldstra, H.: *J. Neurochem.*, 12, 581 (1965)
22) Roberts, E. and Simonsen, D.G.: *Biochem. Pharmacol.*, 12, 113 (1963)
23) Kuriyama, K. and Sze, P.Y.: *Neuropharmacol.*, 10, 103 (1971)
24) Kuriyama, K., Haber, B., Siskin, B. and Roberts, E.: *Proc. natl. Acad. Sci.*, 55, 846 (1966)
25) Rodriguez de Lores Arnaiz, G., Alberici, M., Robiolo, B. and Mistrorigo, M.: *J. Neurochem.*, 21, 615 (1973)