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EFFECT OF CYCLIC ANALOGUES OF VALPROIC ACID ON GLUTAMIC ACID DECARBOXYLASE ACTIVITY AS DETERMINED BY DIFFERENT METHODS*

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Key Word Index Glutamic acid decarboxylase; enzyme inhibition; cyclic analogues of valproic acid; anticonvulsant activity.

Abstract—In order to study the anticonvulsant activity of cyclic analogues of valproic acid, the effects of sodium valproate, sodium spiro (4:6) undecane-2-carboxylate, and sodium spiro (4:6) undecane-2-acetate were observed on the L-glutamic acid decarboxylase (GAD) activity of *E. coli* and *C. welchii* by radioisotopic, volumetric, and gravimetric methods. The results indicated that these compounds potentiated enzyme activity at low concentrations but higher concentrations exhibited an inhibitory effect. Fifty percent enzyme inhibition (ID_{50}) ranges were 200–250 μg/ml, 50–75 μg/ml, and 150–200 μg/ml, for the three compounds respectively as determined by the radioisotopic method. While the radioisotopic was used as a conventional method, the microvolumetric and microgravimetric methods were developed for the first time and found to be applicable in determining the effect of the compounds on enzyme activity. The advantages of the latter two methods are that they are simple, safe, and inexpensive as compared to the radioisotopic method. The results by these methods indicated that the second compound was the strongest inhibitor of GAD activity.

INTRODUCTION

Glutamic acid decarboxylase (GAD) is an enzyme that has been found in the central nervous system of several vertebrate animals. Albers and Brady reported the distribution of this enzyme in different parts of the Rhesus monkey [1]. Other workers reported that GAD was preferentially located in nerve terminals [2]. Furthermore, certain bacteria, such as *E. coli* and *C. welchii*, also contained this enzyme. The occurrence of the enzyme in bacteria as well as higher animals indicated this enzyme plays an important role in biological systems.

GAD decarboxylates L-glutamic acid to form GABA, γ-aminobutyric acid (Fig. 1). GABA is an inhibitory neurotransmitter in brain and controls the neural excitability [3]. It is well established that the impairment of GABA-mediated inhibition leads to convulsions. Some anticonvulsant drugs act either by increasing brain GABA levels or by mimicking or enhancing the inhibitory action of GABA [4]. But for most of the anticonvulsant drugs, the mechanism of action is not known. However, experimental evidence suggests that anticonvulsant agents effect the activity of some enzymes and modulate the levels and functions of GABA in the central nervous system [5].

In our recent publication, we reported synthesis and anticonvulsant evaluation of some spiro analogues of valproic acid [6]. The results indicated that compounds **1** and **2** were effective in pentylenetetrazole- and picrotoxin-induced seizures in mice, while **3** displayed toxicity. The present work was undertaken to study the

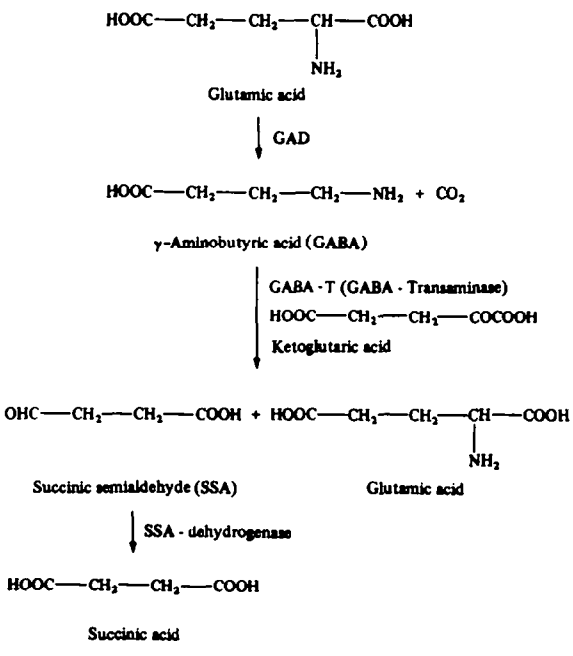

Fig. 1. Some reaction sequences of glutamic acid.

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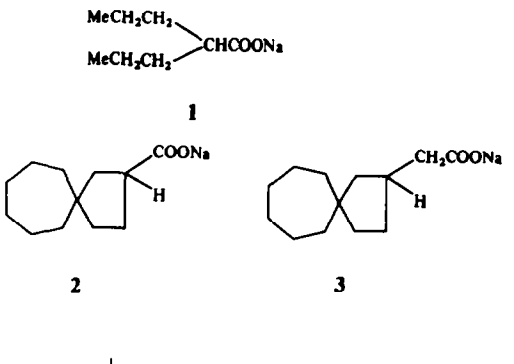
effect of these compounds on the GAD activity *in vitro* and correlate this effect with the anti-convulsant evaluation. The activity was determined by different methods, such as radioactivity, volumetry, and gravimetry. The latter two methods were developed for the first time to correlate the results with the conventional radioactivity method. These methods were applied in studying the effect of cyclic analogues of valproic acid on the GAD activity.



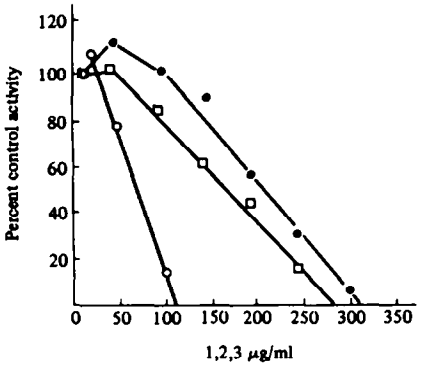


Fig. 2. Effect of 1 (●), 2 (○), and 3 (□) on glutamic acid decarboxylase (GAD) activity in increasing concentrations. Control radioactivity was 50 000 ± 500 c.p.m. An average of three experiments is shown.

RESULTS AND DISCUSSION

Decarboxylation of glutamic acid to GABA involves release of carbon dioxide (Fig. 1). In order to estimate the amount of gas released, different methods have been used in literature. Some of these methods employ manometric [7, 8], radio-isotopic [9], fluorometric [10], spectroscopic [11], and chromatographic [12] methods of estimating carbon dioxide. We used the conventional radioisotopic method but also developed microvolumetric and microgravimetric methods for the estimation of carbon dioxide. The latter two methods are not reported in literature.

By the radioisotopic method, the effect of 1–3 was determined on GAD activity as shown in Table 1. The percentage inhibition of enzyme by 1, 2, and 3 was found to be 23, 70, and 30, respectively. The effect of these compounds on the enzyme activity in increasing concentrations was observed as shown in Fig. 2. The inhibitory curves indicated that there was a slight potentiation effect in the initial concentrations. However, subsequent concentrations exhibited an inhibitory effect on enzyme activity. The mechanism of potentiation effect and inhibition of enzyme activity is not known. Fifty percent enzyme inhibition (ID_{50}) by 1, 2, and 3 was in the ranges of 200–250 μg/ml, 50–75 μg/ml, and 150–200 μg/ml, respectively.

Some of the reactions of the decarboxylation of glutamic acid to GABA and metabolism of this compound to other products are shown in Fig. 1. GABA may react with ketoglutaric acid in the presence of GABA-T (GABA-transaminase) to form SSA (succinic semialdehyde) which on biological oxidation by SSA-dehydrogenase forms succinic acid. Compound 1 was reported to be a weak inhibitor of SSA-dehydrogenase [13, 14] but increased

Table 1. Effect of cyclic analogues of valproic acid 1–3 on glutamic acid decarboxylase activity

	Method*					
Compound (100 mg/ml)	Radioisotopic GAD activity/inhibition (c.p.m.)	(%)	Volumetric† Sodium carbonate/ inhibition (mg)	(%)	Gravimetric‡ Barium carbonate/ inhibition (mg)	(%)
Control	24 000 ± 250		22.00 ± 2.00		22.00 ± 0.25	
1	18 500 + 180	23	16.50 ± 0.85	25	17.30 ± 0.18	22
Control	25 000 ± 260		12.00 ± 0.12		22.00 ± 0.20	
2	7 500 + 75	70	3.50 ± 0.60	71	6.10 ± 0.15	72
Control	26 000 ± 275		12.00 ± 0.12		22.00 + 0.25	
3	18 200 ± 190	30	7.80 ± 0.75	35	14.90 ± 0.15	32

*See Experimental section for enzyme assay and inhibition. Average of three experiments showed ± 5% error.

†The volumetric method was also carried out by absorbing carbon dioxide in a saturated solution of barium hydroxide instead of sodium hydroxide solution and the unreacted base was titrated with standard hydrochloric acid after removing barium carbonate using phenolphthalein as an indicator.

‡The gravimetric method was also carried out by absorbing carbon dioxide in a saturated solution of barium hydroxide. Barium carbonate was removed by centrifugation, washed with distilled water, and weighed.

the levels and metabolism of GABA in mouse [15]. Its anticonvulsant activity in a wide variety of animals provided the rationale for effective use in different types of seizures [16, 17]. However, there is no published report on the effect of 2 and 3 on GAD and other enzyme activities *in vitro*. The present investigation revealed that 1 and 3 were weak inhibitors of GAD activity whereas 2 was a strong inhibitor. Our previously published work on anticonvulsant evaluation of 1–3 indicated that 2 was effective against pentylenetetrazole- and picrotoxin-induced seizures in mice whereas 3 was less effective in such experiments [6]. Since our *in vitro* studies indicated a potentiation effect in initial concentrations and an inhibitory effect in subsequent concentrations, this data could not explain increased levels of GABA and protection of the animals against induced convulsions. Whether the potentiation and/or inhibitory effect(s) can result in the elevation of GABA in the biological system, is not clear at this point. Further work on GABA-T and other degradative enzymes by 1–3 may explain the exact mode of action of these compounds.

By the volumetric method, carbon dioxide in the decarboxylation reaction was absorbed in a known volume of standard sodium hydroxide solution forming a mixture of sodium carbonate and sodium hydroxide. Sodium carbonate, on treatment with barium chloride formed barium carbonate which was not filtered from the reaction mixture. This filtration was not necessary since barium carbonate was sparingly soluble and replaced the strong alkalinity of sodium carbonate due to hydrolysis, thereby, imparting a pH of 8.6 to the solution. Phenolphthalein was, therefore, useful as an indicator in the titration of unreacted sodium hydroxide against standard hydrochloric acid. This titer value represented the content of sodium hydroxide. It was subtracted from the total titer value obtained by titrating an aliquot solution with standard hydrochloric acid using methyl orange as an indicator. This procedure was used to study the effect of 1–3 on GAD activity as shown in Table 1. Sodium carbonate was estimated in the control as well as assay mixture containing inhibitor as described in the experimental section. The percentage enzyme inhibition by 1, 2, and 3 was 25, 71, and 35, respectively. By the gravimetric method, barium carbonate was estimated by two methods: absorbing carbon dioxide on filter paper soaked with barium hydroxide or absorbing the gas directly into the saturated solution of barium hydroxide. The results of these methods are also shown in Table 1. In this case, percent enzyme inhibition by 1, 2, and 3 was 22, 72, and 32, respectively.

The volumetric and gravimetric methods are reported for the first time to estimate carbon dioxide in the biological decarboxylation reaction in microgram quantities. These methods indicate $\pm 3\%$ variation in enzyme inhibition which are comparable to radioactivity method. The volumetric and gravimetric methods are safe, simple, and inexpensive as compared to the radioactivity method.

The decarboxylation reactions were monitored by TLC of the reaction mixture after incubation. L-Glutamic acid and GABA were separated on Whatman KGF gel plates with the solvent systems: pyridine–methanol–water (0.4:2.0:8.0), butanol–acetic acid–water (4.0:1.0:1.0), propanol–water (1.0:1.0), phenol–water (3.0:1.0), alcohol–water (9.0:1.0). Ninhydrin was used as a spray reagent. The R_f values with alcohol–water system were 0.70 (L-glutamic acid) and 0.46 (GABA) indicating that this was a good solvent system. TLC did not show a quantitative relationship of the forward reaction but was helpful in identifying the formation of GABA from the substrate.

EXPERIMENTAL

Materials. Purified L-glutamic acid decarboxylase (GAD) from *E. coli* and *C. welchii* (Sigma Chemical Company, St. Louis, Mo.) had a sp. act. of 0.41 units/mg and 0.80 units/mg protein, respectively. One unit of enzyme from *E. coli* and *C. welchii* released 1.0 μmol CO_2 per min from 35 μmol and 0.80 μmole L-glutamic acid, respectively at 37°. L-[1-^{14}C]Glutamic acid (New England Nuclear, Mass.) had a sp. act. of 49.6 mCi/mmol. Hyamine base (benzethonium chloride) was obtained from Aldrich Chemical Company. Sodium valproate (1) was prepared by neutralizing valproic acid (Saber Laboratories, Inc., Il.) with an equivalent quantity of sodium hydroxide solution and adjusting the solution to pH 7.0. Sodium spiro[4:6]undecane-2-carboxylate (2), and sodium spiro[4:6]undecane-2-acetate (3) were synthesized in our laboratory as reported previously [6]. All other chemicals were of analytical grade.

Apparatus. For the decarboxylation reaction, the apparatus consisted of a two-necked conical flask (10 ml) closed by rubber septa. The mouth of the flask allowed a central well to pass through the septum, and the side arm septum was used for the injection of enzyme by a 1 cm^3 tuberculine syringe. A soln of NaOH or a filter paper soaked in a satd $Ba(OH)_2$ solution was used in the central well in the volumetric and gravimetric methods, respectively. For the radioactivity method, a filter paper soaked in the hyamine base was used.

Methods. Three methods, namely radioactivity, volumetry, and gravimetry were used to estimate the amount of carbon dioxide evolved from the decarboxylation reaction. By the radioactivity method, labelled CO_2 was absorbed in the hyamine base and the radioactivity determined in liquid scintillation fluid. By the volumetric method, CO_2 was absorbed in 0.1 N NaOH and the unreacted base titrated against 0.1 N HCl [18]. Total alkali (carbonate and hydroxide) was determined by titrating with 0.1 N HCl using methyl orange as indicator (V ml). The carbonate was pptd with a slight excess of 1% $BaCl_2$ solution by heating the solution at 70°, cooling and then titrating with 0.1 N HCl using phenolphthalein as indicator (v ml). This volume gave the hydroxide content and by subtracting this volume from the first titration volume, the required volume (V − v) ml of HCl for the CO_3^{2+} was obtained. The results were calculated on the basis that 1 ml of 1 N HCl = 53 mg Na_2CO_3. By the gravimetric method, the paper soaked in a saturated solution of $Ba(OH)_2$ was used and CO_2 was absorbed. The $BaCO_3$ on the paper was dried in an oven at 80° to a constant weight and the results calculated by comparison with the control.

Enzyme assay and inhibition. The reaction mixture contained 0.05 M L-glutamic acid, 0.012 μmol pyridoxal phosphate, 0.013 μmol L-[1-^{14}C]glutamic acid, 0.05 M sodium acetate buffer (pH 5.0), 0.08 μmol sodium chloride, 0.20 ml (0.3–0.5 units) of enzyme from *E. coli* or *C. welchii* and an appropriate quantity of compound. Labelled glutamic acid was omitted in the reaction mixture when the volumetric and gravimetric methods were used in the determination of CO_2. For the control assays, the reaction mixture contained all the above ingredients except compound. The reaction mixture was incubated at 37° in a shaking water bath for 1 hr. The reaction was stopped by chilling the reaction mixture at 4° when the volumetirc and gravimetric methods were used. In case of radioactivity method, the reaction was terminated by adding 0.2 ml of 75% trichloroacetic acid and by further incubating the reaction mixture for 1.5 hr.

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