Formation of γ-Aminobutyric Acid (GABA) in Brain of Mice Treated with L-Glutamic Acid-γ-Hydrazide and Pyridoxal Phosphate-γ-Glutamyl Hydrazone.* (32406)

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The administration of glutamate decarboxylase inhibitors to mice is frequently attended by convulsions. The cause of the convulsion has been ascribed to the drop in the level of GABA in brain often observed in the animals treated with the inhibitors(1,2).

The correlation between levels of GABA in brain and convulsions holds true in some instances but there are many arguments supporting the view that convulsions induced by some compounds are not necessarily related to the total concentration of GABA in brain (for reviews see (3) and (4)). The arguments find ample support in experiments in which convulsions are induced by administering to mice substances causing a decrease in the level of GABA and also substances causing an increase. For example, administration of pyridoxal phosphate-γ-glutamyl hydrazone induces convulsions but the concentration of GABA in brain decreases(5). On the other hand, administration of glutamic acid-γ-hydrazide results in convulsions while the level of GABA in brain actually increases(6,7).

In both instances glutamate decarboxylase is inhibited, which could account for the drop in brain GABA in the first case(5). The increase in the level of GABA in the second case is probably due to a parallel inhibition of GABA transaminase(7), an effect not shared by pyridoxal phosphate-γ-glutamyl hydrazone.

These and other experiments strongly suggest a lack of correlation between GABA concentration in brain and convulsions; they do not rule out, however, the possibility that the rate of GABA formation at the moment of convulsions is a critical factor(3,7). This possibility has been tested in the present experiments by measuring the rate of GABA formation from ^{14}C-labeled glutamate in mice treated with L-glutamic acid-γ-hydrazide and with pyridoxal phosphate-γ-glutamyl hydrazone.

Materials and methods. Yale Swiss mice (22-29 g) were used in all the experiments. L-glutamic acid-γ-hydrazide was obtained from Cyclo Chemical Co. Pyridoxal phosphate-γ-glutamyl hydrazone was synthesized as previously reported(5). DL-glutamic acid-3,4-^{14}C was obtained from CalBiochem or from Volk (specific activity 8.5-10 mc/mM). Its purity was checked by chromatography.

The mice were injected intraperitoneally with glutamic acid-γ-hydrazide (2 g/kg) or pyridoxal phosphate-γ-glutamyl hydrazone (80 mg/kg) (control mice were injected with 0.9% saline solution). At the time indicated under *Results*, 1 μc of radioactive glutamic acid was injected intracranially, without anesthesia, in the medial suture, behind the occipital protuberance, at an angle of about 45°, using a ¼″ 27 gauge needle and a microsyringe. The volume injected was 0.01 ml. Some mice showed symptoms of cerebellar lesion, manifested by fast rotary movements of the body and circular walking. However, control mice showed no alteration in the levels of glutamic acid or GABA in brain as compared with intact animals. Two minutes were allowed in some experiments between the injection of labeled glutamic acid and the time the animals were killed. In other experiments a time of 6 minutes was allowed (see *Results*).

Separation of amino acids and radioactivity measure. The mice were decapitated and the brain, without cerebellum, pons and medulla, was removed and frozen in a dry ice-acetone mixture. The time between decapitation and freezing of the tissue was 30-45 sec. The brain was weighed while frozen and homogenized in 4 ml of 10% TCA +

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2 ml to rinse the homogenizer. The homogenates were centrifuged at 2,500 rpm for 15 min and the precipitates washed with 2 ml of 1% TCA. The pH of the supernatants was adjusted to pH 3.5-4 with 10% KOH and passed through a Dowex-50-H^+ column (4 cm $\times$ 1 cm) to eliminate organic acids. The column was washed with 7-8-fold its volume with water and the amino acids were eluted with 16 ml of N NH_4OH. The eluates were evaporated to dryness under an infrared lamp and a stream of hot air. The residue was resuspended in 0.1 ml of water per 200 mg of original tissue, and 20 μl were spotted on Whatman 3MM chromatographic paper, in duplicate. The chromatograms were run (descendent chromatography, 80% phenol), dried for 24 hours and treated with 0.02% ninhydrin in butanol to locate glutamic acid and GABA. The spots corresponding to these two amino acids were cut and the whole piece of paper (about 3 cm $\times$ 6 cm) was put inside scintillation counting vials, in contact with the inner wall. 15 ml of Liquifluor 1X (4 g of 2,5-diphenyloxazole and 50 mg of p-bis(2-(5-phenyloxazol))-benzene per liter of toluene, Nuclear Chicago) were added, and the radioactivity was counted in an automatic Nuclear Chicago scintillation counter. The background activity was subtracted from all values.

Several experiments showed that the orientation of the paper inside the vial only slightly modified the counting, in agreement with reported data(8,9) and that the quenching produced by the ninhydrin reaction color was less than 5% when 0.02% ninhydrin was used. The size of the paper, which was not identical for each spot, had no noticeable effect on the counting. The recovery of radioactivity added to brain homogenates by the procedure employed was 34-38% (without correction for the efficiency of the counter, which was about 40% by this procedure). The maximum error in the linearity of measured radioactivity when different amounts of radioactivity were added to the homogenates was 4%. The results are expressed as percent radioactivity in GABA relative to that in glutamic acid.

Determination of GABA and glutamate. GABA and glutamate were determined in the resuspended residue after evaporating to dryness the eluates from the Dowex-50 column. GABA was determined by the method of Jakoby and Scott(10) as described by Jakoby(11) (the enzymatic preparation used was obtained from Worthington Biochemical Co.) and glutamate by the method described by Bernt and Bergmeyer(12). By these procedures the recovery was 93-99% for glutamic acid and 94-100% for GABA.

Results and discussion. The graphs of Fig. 1 show that the rate of GABA formation from injected glutamate-3,4-^{14}C is less in mice treated with glutamic acid-γ-hydra-

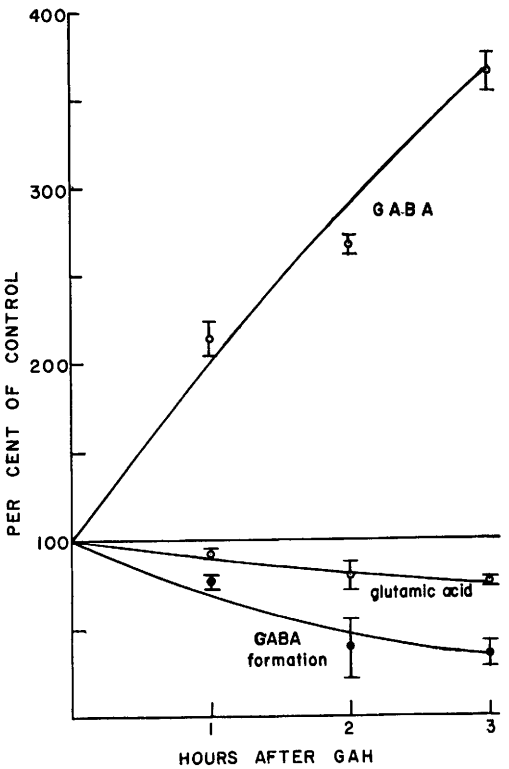

FIG. 1. Changes with time of GABA and glutamic acid levels and of rate of formation of GABA from labeled glutamic acid, in brain of mice treated with L-glutamic acid-γ-hydrazide (2 g/kg). The percent radioactivity in GABA in ralation to that in glutamic acid per 40 mg of tissue, 2 min after intracranial injection of 1 μc glutamic acid-3,4-^{14}C at times indicated, was taken as GABA formation. Each point represents the mean value obtained from 4 to 8 animals, $\pm$ S.E.M. The mean concentrations of GABA and glutamic acid for the 100% levels were (μmoles/g): for GABA, 2.48 (1 hr), 3.29 (2 hr) and 2.74 (3 hr); for glutamic acid, 12.53 (1 hr), 14.51 (2 hr) and 12.15 (3 hr).

zide than in control mice. Convulsions occur at the moment when the formation of GABA is at a minimal rate. This observation is in agreement with previous data, which showed that glutamate decarboxylase activity in brain of mice treated with glutamic acid-γ-hydrazide is maximally inhibited at the moment convulsions occur(7). It is of interest, however, that the concentration of GABA in the whole brain is considerably increased in the treated animals at the moment of occurrence of convulsions. This is interpreted to mean that the site of GABA formation in the brain is a factor of prime importance.

Salganicoff and De Robertis(13) and Balázs *et al*(14) have located glutamic decarboxylase mainly in nerve endings and GABA transaminase mainly in mitochondria. On the basis of the distribution of the two enzymes it can be postulated that the critical site of GABA synthesis is probably the synaptic cleft. Such a hypothesis has been proposed by Wood *et al*(15) on the basis of experiments showing that GABA protects animals against convulsions induced by oxygen at high pressure or by the administration of thiosemicarbazide.

The effect of pyridoxal phosphate-γ-glutamyl hydrazone on the rate of GABA formation is also inhibitory, as shown in Table I. Moreover, the inhibition is significantly greater at 6 minutes than at 2 minutes after the injection of glutamate-3,4-^{14}C. In contrast, the degree of inhibition of GABA formation by administered glutamic acid-γ-hydrazide is about the same at 2 or 6 minutes after the injection of labeled glutamate. This is what one would expect because glutamic acid-γ-hydrazide also inhibits GABA transaminase(7); this inhibition would result in the accumulation of radioactive GABA produced from the injected radioactive glutamate. Pyridoxal phosphate-γ-glutamyl hydrazone only inhibits glutamate decarboxylase and its net effect would be a pronounced decrease in the rate of GABA formation. The effect of the two compounds on the concentration of GABA in brain is more readily seen in Table II.

The results presented here, supported by previously reported data, favor strongly the idea that inhibition of GABA formation is one if not the most important factor in the induction of convulsions by certain compounds. The concentration of GABA in the whole brain appears not to be a factor in convulsions.

Summary. The effect *in vivo* of L-glutamic acid-γ-hydrazide and of pyridoxal phosphate-γ-glutamyl hydrazone on rate of formation of γ-aminobutyric acid (GABA) from labeled glutamate in mice brain was studied. It was found that both substances inhibited GABA formation and that this inhibition was maximal at the moment convulsions occur. Since the total concentration of GABA in brain is increased by glutamic acid-γ-hydrazide but decreased by pyridoxal phosphate-γ-glutamyl

TABLE I. GABA Formation from Glutamic Acid-3,4-^{14}C *in vivo*, in Brain of Mice Treated with L-Glutamic Acid-γ-Hydrazide or Pyridoxal Phosphate-γ-Glutamyl Hydrazone.

Treatment	Time studied, min*	Control	Treated	% Inhibition
L-glutamic acid-γ-hydrazide (2 g/kg 3 hr before the intracranial injection of radioactive glutamate)	2	1.82 ± .12 (6)	.66 ± .15‡ (6)	63.7
	6	4.45 ± .46† (5)	1.86 ± .35‡ (3)	58.2
Pyridoxal phosphate-γ-glutamyl hydrazone (80 mg /kg 25 min before the intracranial injection of radioactive glutamate)	2	1.42 ± .10 (3)	.89 ± .10§ (6)	37.3
	6	4.45 ± .46† (5)	1.29 ± .04‡ (3)	71.0

The values are % radioactivity in GABA relative to that in glutamic acid per 40 mg of tissue. Mean ± S.E.M. Number of animals in parentheses.

* Time between intracranial injection of labeled glutamic acid (1 μc) and sacrifice of animals.

† Same control group.

‡ $p < .001$ (difference from control values). § $p < .05$ (difference from control values).

TABLE II. GABA Levels in Brain of Mice Treated with L-Glutamic Acid-γ-Hydrazide or Pyridoxal Phosphate-γ-Glutamyl Hydrazone.

Treatment	Control	Treated	% Change
L-glutamic acid-γ-hydrazide (2 g/kg 3 hr before sacrifice)	2.74 ± .31 (8)	10.01 ± .33* (7)	+265
Pyridoxal phosphate-γ-glutamyl hydrazone (80 mg/kg 27 min before sacrifice)	3.56 ± .10 (4)	2.34 ± .09* (6)	— 34.3

The values are μmoles/g ± S.E.M. Number of animals in parentheses.
* $p < .001$ (difference from control values).

hydrazone, it is suggested that the rate of GABA formation, independently of its total concentration, is probably a factor in the production of some kinds of convulsions. It is also suggested that the site of GABA formation in brain appears to be a critical one, and that this critical site might be the synaptic cleft.

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Marburg Immunologic Reactivity of Haptoglobin: Evidence for a Beta-Chain Mutation.* (32407)

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The presence of several atypical types of human serum haptoglobin such as Hp 2-1 Johnson(1), 1-P, 2-P, 1-H, 2-L(2), Hp Ab (3) and Hp 2-1 D(4) have been reported by several investigators. These atypical types can be easily differentiated from the common three phenotypes, *i.e.*, 1-1, 2-1 and 2-2, by ordinary starch gel electrophoresis after addition of hemoglobin.

In addition, the existence of immunologically atypical haptoglobins which show normal starch gel electrophoretic patterns was also recognized by Korngold(5) and Shim and Bearn(6). The nature of these atypical haptoglobin molecules has not been clarified.

Haptoglobin Marburg (Hp Mb), thus far,

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