Biochemical Pharmacology, Vol. 16, pp. 1211–1218. Pergamon Press Ltd. 1967. Printed in Great Britain

MODIFICATIONS OF BRAIN GLUTAMATE DECARBOXYLASE ACTIVITY BY PYRIDOXAL PHOSPHATE-γ-GLUTAMYL HYDRAZONE*

RICARDO TAPIA, MIGUEL PÉREZ DE LA MORA and GUILLERMO H. MASSIEU

Departmento de Bioquímica, Instituto de Biología, Universidad Nacional de México, México D.F., México

(*Received* 2 *November* 1966; *accepted* 17 *January* 1967)

Abstract—The administration of pyridoxal phosphate-γ-glutamyl hydrazone to mice had effects identical with those produced by the simultaneous administration of equimolar doses of glutamic acid-γ-hydrazide and pyridoxal phosphate; both treatments produced convulsions, lowered the brain γ-aminobutyric acid concentration, and inhibited the activity of glutamate decarboxylase. The inhibition was completely reversed when pyridoxal phosphate was added *in vitro* to the incubation mixture. Pyridoxal phosphate-γ-glutamyl hydrazone administration also inhibited the brain pyridoxal kinase activity. On the other hand, the addition of pyridoxal phosphate-γ-glutamyl hydrazone *in vitro* to brain homogenates resulted in an activation of glutamate decarboxylase similar to that obtained with free pyridoxal phosphate.

It is concluded that the effects of the simultaneous administration of glutamic acid-γ-hydrazide and pyridoxal phosphate are due to pyridoxal phosphate-γ-glutamyl hydrazone, which is formed *in vivo*, and that such effects are possibly mediated by the inhibition of brain pyridoxal kinase. Rupture of pyridoxal phosphate-γ-glutamyl hydrazone at the level of the apoenzyme active site apparently occurs when the substance is added *in vitro*.

IN A PREVIOUS work[1] it was shown that the simultaneous administration of glutamic acid-γ-hydrazide (γ-glu·$NHNH_2$)† and pyridoxal phosphate (pyridoxal-P) to mice induces fatal convulsions at a time when glutamate decarboxylase (GAD) activity and γ-aminobutyric acid (GABA) concentration in brain were notably diminished. The injection of γ-glu·$NHNH_2$ alone does not induce these effects; rather it produces an increase in GABA levels, owing to a more intense inhibition of aminobutyrate aminotransferase (ABAT) activity,[2,3] although very high doses of γ-glu·$NHNH_2$ induce a more remarkable inhibition of the former enzyme activity.[4] Since this hydrazide is a powerful GAD inhibitor *in vitro*,[3] it was previously suggested[1] that pyridoxal-P might facilitate the entrance of γ-glu·$NHNH_2$ into the neurons, thus permitting the inhibition of GAD. However, it is known that some hydrazides inhibit GAD activity as a result of their interaction with the formyl groups of pyridoxal-P;[5] some pyridoxal hydrazones also produce convulsions,[6] whereas others strongly inhibit pyridoxal

* A summary of this work was presented during the 1966 Fall Meeting of the American Society for Pharmacology and Experimental Therapeutics held in Mexico City, Mexico (July 15–20).

† The abbreviations used in this work are: γ-glu·$NHNH_2$, L-glutamic acid-γ-hydrazide; pyridoxal-P, pyridoxal phosphate; GAD, glutamate decarboxylase (EC 4.1.1.15); ABAT, aminobutyrate aminotransferase (EC 2.6.1.19); GABA, γ-aminobutyric acid; AOP, amino-oxypropionic acid; AOA, amino-oxyacetic acid.

kinase activity in brain;[7] and some pyridoxal-P-hydrazones are formed *in vivo*.[8,9] Therefore, the possibility that the effects of γ-glu·$NHNH_2$ plus pyridoxal-P treatment are due to the formation of pyridoxal phosphate-γ-glutamyl hydrazone seemed to be worthy of consideration.

In the present paper it is reported that pyridoxal phosphate-γ-glutamyl hydrazone has effects identical with those exhibited by the simultaneous administration of γ-glu·$NHNH_2$ and pyridoxal-P. It is also shown that the simultaneous injection of pyridoxal-P and several other potent GAD inhibitors did not modify mice behaviour or their free amino acid content of brain.

MATERIALS AND METHODS

Adult nonfasted mice (local strain) weighing 23–30 g were used throughout the experimental work. Substances were obtained as follows: pyridoxal-P and pyridoxal hydrochloride from Nutritional Biochemicals Co.; deoxypyridoxine phosphate, L-glutamic acid-γ-ethyl ester, and γ-glu·$NHNH_2$, from Calbiochem; ATP from Sigma Chemical Co.; and hydroxylamine from Eastman Kodak. AOA (hemihydrochloride) and AOP were synthesized by Dr. Guillermo Carvajal. Pyridoxal phosphate-γ-glutamyl hydrazone was prepared by following the procedure of Sah[10] with slight modifications. An aliquot was run on paper chromatography (80 per cent phenol) to ascertain its purity; only one u.v. and ninhydrin-positive spot with a 0·31 R_f was obtained. The absorption spectrum of pyridoxal phosphate-γ-glutamyl hydrazone in 0·1 M phosphate buffer, pH 6·3, showed a maximum at 286 mμ and another ill-defined maximum of lower optical density at 340 mμ. This spectrum is similar to that reported for pyridoxal-P-carbohydrazone and pyridoxal-P-semicarbazone.[11]

GAD activity was estimated by measuring CO_2 production by the conventional Warburg manometric technique, after a 10-min equilibrium period without substrate and under nitrogen atmosphere at 37°. The incubation time in all cases was 1 hr; measurements were taken every 10 min. The incubation mixture contained 0·05 M phosphate buffer (pH 6·3), 0·033 M glutamic acid neutralized to pH 6·3, and 240 mg brain tissue homogenized in double-distilled water (1:4 w/v). When added, pyridoxal-P and pyridoxal phosphate-γ-glutamyl hydrazone concentrations are indicated under Results. Glutamate was added from the side arm in a volume of 0·35 ml. The total volume in each Warburg flask was 3·2 ml.

Pyridoxal kinase activity was measured by preincubating for 30 min at pH 6·3 in an incubation mixture containing: 0·5 mM ATP, 0·5 mM pyridoxal, 0·05 mM zinc sulfate, and 1·2 ml of a 30-min., 18,000-*g* supernatant of a Triton-X-100 homogenate of brain tissue. The brain tissue was ground in a Potter-Elvehjem homogenizer with a Triton-X-100 solution (1:4 w/v) in such a way that the concentration of the detergent in the homogenate was 0·5%. It has been shown that, under similar conditions of incubation, brain pyridoxal kinase is active[7,12] and GAD activity is more sensitive to pyridoxal-P than is the case with water or buffer homogenates.[13] After this preincubation period, glutamic acid was added from the side arm to the main compartment of the Warburg flasks, and GAD activity was measured for 1 hr, as described above. However, since GAD activity is strongly inhibited by pyridoxal phosphate-γ-glutamyl hydrazone treatment, the following procedure was used in order to measure the effect *in vivo* of pyridoxal phosphate-γ-glutamyl hydrazone on pyridoxal kinase activity. The brains of 4 control mice or of the 4 pyridoxal phosphate-γ-glutamyl hydrazone-treated

mice (205 μmole/kg) were pooled in order to obtain enough supernatant fluid for three Warburg flasks. The first flask contained ATP, pyridoxal, and zinc sulfate at the concentrations already indicated; the second contained pyridoxal-P at $1{\cdot}26 \times 10^{-5}$ M; the third did not contain pyridoxal kinase substrates or pyridoxal-P. The activity of pyridoxal kinase was then calculated as the ratio: percentage of GAD activation by preincubation with ATP and pyridoxal/percentage of GAD activation by $1{\cdot}26 \times 10^{-5}$ M pyridoxal-P.

It has been shown that AOA, AOP, and hydroxylamine, like γ-glu·$NHNH_2$, strongly inhibit GAD activity *in vitro*.[14] Therefore, pyridoxal-P at a dose of 50 mg/kg was injected simultaneously with AOA, AOP, or hydroxylamine, and the mice were sacrificed some time after the administration of the drugs. The dose of the substance and the time of sacrifice after treatment were as follows: AOA, 50 mg/kg, 120 min; AOP, 120 mg/kg, 30 min; hydroxylamine, 50 mg/kg, 75 min; L-glutamic acid-γ-ethyl ester, 1500 mg/kg, 60 min. The last substance named has been observed to have no effect on free amino acids in brain (unpublished work) and it seemed of interest to determine whether the simultaneous administration of pyridoxal-P could make it effective. In all cases aspartic acid, glutamic acid, glutamine, and GABA concentrations in brain were measured at the time indicated. Brains of control mice injected either with pyridoxal-P or AOA, AOP, or hydroxylamine alone, or with saline solution, were assayed for these amino acids also. The amino acids mentioned were separated and measured by the previously described chromatographic and colorimetric methods[3,15] in which 90–95 per cent recovery of amino acids is obtained.[15,16]

In other experiments deoxypyridoxine phosphate (47 mg/kg) was injected simultaneously with γ-glu·$NHNH_2$ (80 mg/kg) or with γ-glu·$NHNH_2$ plus pyridoxal-P, and the free amino acids in the brain of these mice were measured as described above.

RESULTS

Effects of pyridoxal phosphate-γ-glutamyl hydrazone

All mice treated with equimolar doses of γ-glu·$NHNH_2$ plus pyridoxal-P or with pyridoxal phosphate-γ-glutamyl hydrazone died in convulsions at the same time. The inhibition of brain GAD activity and the decrease of GABA concentration at the moment of convulsion were also similar in both groups (about 60 per cent). GAD inhibition was completely reversed in both groups by the addition of pyridoxal-P to the incubation mixture (Table 1). In other experiments it was observed that neither pyridoxal-P by itself nor γ-glu·$NHNH_2$ alone diminished GAD activity *in vivo* at the dose employed (205 μmole/kg). At lower equimolar doses of γ-glu·$NHNH_2$ plus pyridoxal-P or pyridoxal phosphate-γ-glutamyl hydrazone (110 μmole/kg), about 33 per cent of the 16 animals injected died in convulsions.

Some experiments were done in which AOA at the dose of 50 mg/kg was injected 90 min before the administration of pyridoxal phosphate-γ-glutamyl hydrazone. It was observed that this acid prevented the convulsions produced by the hydrazone, just as it prevented convulsions induced by γ-glu·$NHNH_2$ plus pyridoxal-P treatment.[4]

When pyridoxal phosphate-γ-glutamyl hydrazone was added *in vitro* to brain homogenates at 10^{-4} M or higher concentrations, an activation of GAD similar to that produced by the same equimolar concentrations of pyridoxal-P was observed (Fig. 1). This fact suggested that, *in vitro*, pyridoxal phosphate-γ-glutamyl hydrazone is split,

TABLE 1. GABA CONCENTRATION AND GAD ACTIVITY IN BRAIN OF CONTROL MICE AND OF MICE TREATED WITH γ-GLU·$NHNH_2$ + PYRIDOXAL-P (205 μMOLE/KG EACH) AND PYRIDOXAL-P-γ-GLUTAMYL HYDRAZONE (205 μMOLE/KG)

Group	GABA* (mg/100 g)	GAD† (μl CO_2/hr)	
		Without pyridoxal-P	With pyridoxal-P‡
Control	44·2 ± 1·60* (8)	137	234
γ-Glu·$NHNH_2$ + pyridoxal-P (12/12, 41 min)§	17·0 ± 0·96 [61·5]¶ (5)	51 [62·8]	230
Pyridoxal-P-γ-glutamyl hydrazone (12/12, 38 min)§	16·8 ± 0·90 [62·0] (8)	57 [58·5]	239

* The figures are mean ± standard error. Number of animals in parentheses.

† Incubation conditions as described under Methods. Data from a typical experiment repeated 3 times.

‡ Final concentration: $1·26 \times 10^{-4}$ M.

§ Number of mice dead in convulsions/number of treated mice, and mean time of death in convulsions.

¶ Percent of decrease in comparison with control animals, in brackets.

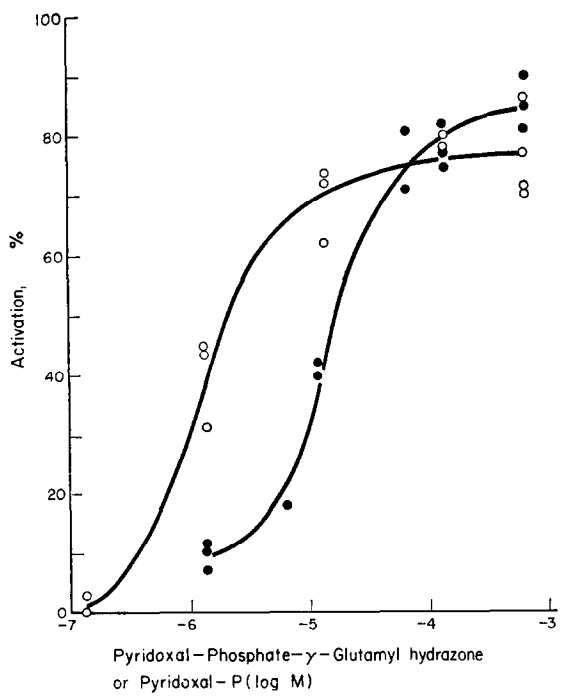

FIG. 1. Activation of GAD by different concentrations of pyridoxal-P (○) or pyridoxal phosphate-γ-glutamyl hydrazone (●). One hr of incubation under the conditions described in Methods.

yielding pyridoxal phosphate and γ-glu·$NHNH_2$, and that under these conditions pyridoxal-P activates GAD. However, since the hydrazide alone, when added *in vitro* to brain homogenates, inhibits GAD activity,[3] some experiments were done in which γ-glu·$NHNH_2$ and pyridoxal-P were added at the molar concentrations that would result from hydrazone rupture. Under these conditions GAD was activated to the same extent as when pyridoxal-P alone was added, whereas the hydrazide alone at the same concentration inhibited the enzyme (Fig. 2). Thus it is indeed possible that

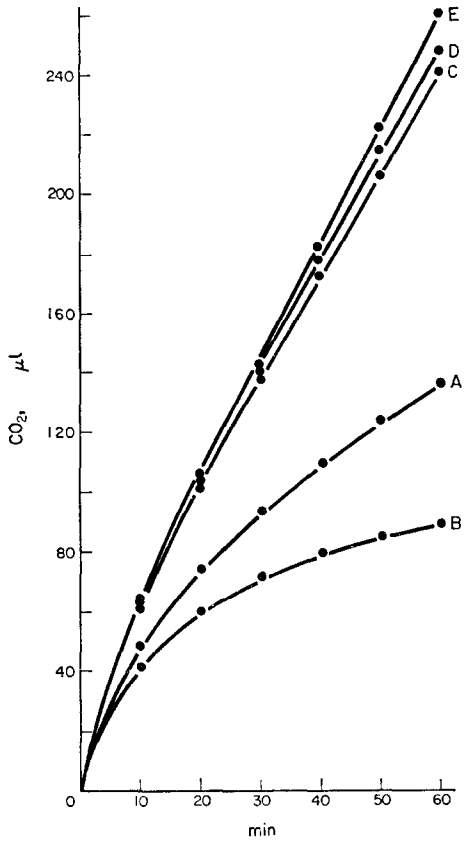

FIG. 2. GAD activity without any addition (curve A) and in the presence of γ-glu·NHNH; (curve B), γ-glu·$NHNH_2$ plus pyridoxal-P (curve C), pyridoxal-P (curve D), or pyridoxal phosphate-γ-glutamyl hydrazone (curve E). All substances were added at $1{\cdot}26 \times 10^{-4}$ M final concentration. Incubation conditions are described in Methods.

the activation of GAD by pyridoxal phosphate-γ-glutamyl hydrazone *in vitro* is due to pyridoxal-P which results from hydrazone.

The effect of pyridoxal phosphate-γ-glutamyl hydrazone administration on brain pyridoxal kinase is shown in Table 2. It can be seen that at the moment of convulsion the enzyme is 55 per cent inhibited.

Simultaneous administration of pyridoxal-P and several substances

The changes in the concentration of the amino acids studied after the administration of pyridoxal-P (a decrease of GABA levels) and after the injection of AOA (decrease of

aspartic and glutamic acids and a notable increase of GABA levels) have been reported.[1,17–19] The simultaneous administration of pyridoxal-P produced no important differences in the reported amino acid changes. AOP, L-glutamic acid-γ-ethyl ester, and hydroxylamine did not affect significantly the levels of the amino acids studied, either when administered alone or when injected simultaneously with pyridoxal-P. In

TABLE 2. EFFECT OF PYRIDOXAL PHOSPHATE-γ-GLUTAMYL HYDRAZONE TREATMENT (205 μMOLE/KG) ON PYRIDOXAL KINASE ACTIVITY IN MOUSE BRAIN*

	Control mice	Treated mice	Inhibition (%)
Pyridoxal kinase activity†	0·563 ± 0·054 (4)	0·253 ± 0·039‡ (4)	55·2

* The brains of 4 mice were extracted at the moment of maximum convulsions (about 40 min. after treatment) and pooled for each determination of pyridoxal kinase activity, as described under Methods, Control mice were injected with saline solution.

† Results are expressed as the ratio: per cent of GAD activation by preincubation with ATP and pyridoxal/per cent of GAD activation by $1{\cdot}26 \times 10^{-5}$ M pyridoxal-P, as described under Methods. The values are means ± S.E.M. Number of determinations in parentheses.

‡ Statistical significance of the difference according to t test $P < 0{\cdot}01$.

no case were alterations observed in the behavior of the animals. In the experiments with deoxypyridoxine phosphate and γ-glu·$NHNH_2$, only the previously reported effects of γ-glu·$NHNH_2$ on free amino acids in brain[2] were observed, without alteration in the behavior of mice. Deoxypyridoxine phosphate did not prevent convulsions or the decrease of GABA induced by the simultaneous administration of γ-glu·$NHNH_2$ and pyridoxal-P.

DISCUSSION

It can be concluded from the results of the present paper that the effects of the simultaneous administration of γ-glu·$NHNH_2$ and pyridoxal-P are due to the formation of pyridoxal phosphate-γ-glutamyl hydrazone *in vivo*. This conclusion is based on the fact that equimolar doses of γ-glu·$NHNH_2$ plus pyridoxal-P or pyridoxal phosphate-γ-glutamyl hydrazone had identical effects in relation to: (a) the production of convulsions, (b) the decrease of GABA levels in brain, and (c) the inhibition of GAD activity (reversible by the addition of pyridoxal-P). Furthermore, pyridoxal-P had no effect on these parameters when it was injected simultaneously with several potent GAD inhibitors such as AOA, AOP, and hydroxylamine. The simultaneous injection of γ-glu·$NHNH_2$ and deoxypyridoxine phosphate similarly showed no apparen effect. On the other hand, the anticonvulsant action of AOA on pyridoxal phosphate-γ-glutamyl hydrazone-induced convulsions was also observed with the convulsions produced by γ-glu·$NHNH_2$ plus pyridoxal-P treatment.[4]

It would seem important to mention that the results obtained with pyridoxal phosphate-γ-glutamyl hydrazone agree with the idea[4,20] that the inhibition of GAD activity is related to an increase in brain excitability.

A second conclusion can be drawn from the data presented here, namely, that GAD inhibition induced by pyridoxal phosphate-γ-glutamyl hydrazone administration seems to be secondary to the inhibition of pyridoxal kinase activity. In fact, a significant

inhibition of this enzyme ($P < 0{\cdot}01$, Table 2) was observed after pyridoxal phosphate-γ-glutamyl hydrazone treatment; furthermore, the addition of pyridoxal-P *in vitro* completely reversed the inhibition of GAD produced by pyridoxal phosphate-γ-glutamyl hydrazone administration (Table 1). This fact could be related to the results of McCormick *et al.*,[12] who did not observe GAD inhibition when pyridoxal kinase was inhibited by the dihydrazone of pyridoxal administration, since they added pyridoxal-P to the incubation mixture at a concentration capable of reversing GAD inhibition.

The activation of pyridoxal phosphate-γ-glutamyl hydrazone *in vitro*, as already suggested, seems to be due to pyridoxal-P derived from hydrazone rupture at the level of the apoenzyme active site according to the reaction: GAD + pyridoxal phosphate-γ-glutamyl hydrazone $\rightleftarrows$ GAD-pyridoxal-P + γ-glu·$NHNH_2$

The experiments of Torchinsky[21] and Fujioka and Snell[22] on the action of carbonyl reagent derivatives of pyridoxal on the activity of aminotransferases support this hypothesis. Gonnard *et al.*[23,24] and Makino *et al.*[25] claim that some pyridoxal-P hydrazones as such might act as coenzymes of GAD and of other pyridoxal-P enzymes. However, their experiments may be interpreted on the basis of other available data,[21,22] which indicate that the activating effect of these hydrazones is due to pyridoxal-P. In this regard it is noteworthy that Makino *et al.*[25] used trichloroacetic acid for precipitating proteins after incubating pyridoxal-P-isonicotinoyl hydrazone with a preparation of GAD. They found the hydrazone but no free pyridoxal-P in the supernatant fluid. However, it has been found in our laboratory (unpublished results) that at strongly acid pH (in presence of trichloroacetic acid), γ-glu·$NHNH_2$ and pyridoxal-P readily interact to form the hydrazone. This reaction could also occur with isoniazid and pyridoxal-P in Makino's experiments.

Acknowledgements—The authors wish to thank Dr. Guillermo Carvajal for furnishing the AOA and AOP used in this work.

REFERENCES

1. G. H. Massieu, R. I. Tapia, H. Pasantes and B. G. Ortega, *Biochem. Pharmac.* **13**, 118 (1964).
2. G. H. Massieu, R. I. Tapia and B. G. Ortega, *Biochem. Pharmac.* **11**, 976 (1962).
3. R. I. Tapia, H. Pasantes, B. G. Ortega and G. H. Massieu, *Biochem. Pharmac.* **14**, 1831 (1966).
4. R. I. Tapia, H. Pasantes, M. Pérez de la Mora, B. G. Ortega and G. H. Massieu, *Biochem. Pharmac.* in press.
5. A. N. Davison, *Biochim. biophys. Acta* **19**, 131 (1956).
6. B. Dubnick, G. A. Leeson and C. C. Scott, *Toxic. appl. Pharmac.* **2**, 403 (1960).
7. D. B. McCormick and E. E. Snell, *Proc. natn. Acad. Sci. U.S.A.* **45**, 1371 (1959).
8. H. L. Williams and D. H. Abdulian, *J. Pharmac. exp. Ther.* **116**, 62 (1956).
9. J. A. Bain and H. L. Williams, in *Inhibition in the Nervous System and Gamma-aminobutyric Acid* (Ed. E. Roberts), p. 275. Pergamon Press, Oxford (1960).
10. P. Sah, *J. Am. chem. Soc.* **76**, 300 (1954).
11. R. G. Wiegand, *J. Am. chem. Soc.* **78**, 5307 (1956).
12. D. B. McCormick, B. M. Guirard and E. E. Snell, *Proc. Soc. exp. Biol. Med.* **104**, 554 (1960).
13. R. Tapia, H. Pasantes, J. Márquez and G. H. Massieu, submitted for publication.
14. E. Roberts and D. G. Simonsen, *Biochem. Pharmac.* **12**, 113 (1963).
15. G. Carvajal, M. Russek, R. Tapia and G. Massieu, *Biochem. Pharmac.* **13**, 1059 (1964).
16. G. H. Massieu, *An. Inst. Biol. Univ Méx.* **29**, 9 (1958).
17. P. Dante Roa, J. K. Tews and W. E. Stone, *Biochem. Pharmac.* **13**, 477 (1964).
18. K. Kuriyama, E. Roberts and M. K. Rubinstein, *Biochem. Pharmac.* **15**, 221 (1966).
19. D. P. Wallach, *Biochem. Pharmac.* **5**, 323 (1960).

20. J. D. WOOD, W. J. WATSON and N. E. STACEY, *J. Neurochem.* **13**, 361 (1966).
21. Y. M. TORCHINSKY, *Biochem. biophys. Res. Commun.* **10**, 401 (1963).
22. M. FUJIOKA and E. E. SNELL, *J. biol. Chem.* **240**, 3044 (1965).
23. P. GONNARD and S. FENARD, *J. Neurochem.* **9**, 135 (1962).
24. P. GONNARD, J. DUHAULT, M. CAMIER, C. NGUYEM-PHILIPPON and N. BOIGNE, *Biochim. biophys Acta* **81**, 548 (1964).
25. M. MAKINO, Y. OOI, M. MATSUDO and T. KURODA, in *Chemical and Biological Aspects of Pyridoxal Catalysis* (Eds. E. E. SNELL *et al.*), p. 291. Pergamon Press, Oxford (1963).