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EFFECTS OF VARIOUS SUBSTITUTED HYDRAZONES AND HYDRAZINES OF PYRIDOXAL-5′-PHOSPHATE ON BRAIN GLUTAMATE DECARBOXYLASE*

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Abstract—In addition to pyridoxal-5′-phosphate hydrazone (I), the following substituted hydrazones of pyridoxal-5′-phosphate were prepared: L-glutamyl-γ-hydrazone (II), isonicotinylhydrazone (III) and thiosemicarbazone (IV). The pyridoxal-5′-phosphate oxime-*O*-acetic acid was also prepared. The substituted hydrazines corresponding to I–IV and the reduced oxime-*O*-acetic acid were prepared by reduction of the hydrazones with sodium borohydride.

All the hydrazones and the oxime were tested against glutamate decarboxylase (GAD) from mouse brain. The hydrazones were found to have coenzymatic activity corresponding to 62–87 per cent of the activity measured for pyridoxal phosphate under identical assay conditions. The coenzymatic activity of the four derivatives was in the following order: III > II > I > IV. The oxime-*O*-acetic acid not only was devoid of coenzymatic activity, but it actually inhibited GAD activity to the extent of 50 per cent. The substituted hydrazines tested under identical conditions had no coenzymatic or inhibitory effect. The reduced oxime-*O*-acetic acid also inhibited GAD activity.

Injected i.p. I and II caused fatal convulsions and produced a notable decrease in brain GAD if assayed without pyridoxal phosphate. III had a slight inhibitory effect on GAD and no convulsant action. With added pyridoxal 5′-phosphate, the activity was about the same as that recorded for control animals. The thiosemicarbazone, the oxime, and all the substituted hydrazines had little or no effect on GAD activity under these conditions and they did not produce convulsions.

I, II and III inhibited brain pyridoxal kinase to some extent. Injected intraperitoneally, only I and II had a significant (30–36 per cent) inhibitory effect on brain pyridoxal kinase. Of the substituted hydrazines, only the one corresponding to II had a slight (9 per cent) inhibitory effect.

The results are discussed in relation to the mechanism of activation of GAD *in vitro* by the hydrazones, and attempts are made to correlate the convulsant action of I and II with their effect *in vivo* upon GAD and pyridoxal kinase.

THE ROLE of pyridoxal-5′-phosphate as a coenzyme for amino acid decarboxylases and other enzymes has been studied over the past years, and it has been established that the participation of the carbonyl group of the molecule is essential for its coenzymatic action.[1, 2] However, several authors have found that some derivatives of pyridoxal-5′-phosphate, mainly hydrazones, activate brain GAD‡ (L-glutamate 1-carboxy-lyase, EC 4.1.1.15)[3–6] and other pyridoxal phosphate enzymes.[4, 7–10] Studies on the stability of these derivatives have been interpreted to mean that the

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‡ Abbreviation used: GAD, glutamate decarboxylase.

activation of the enzymes does not necessarily imply the participation of the free carbonyl group.[4-6] Other data in the literature, however, suggest that in the presence of the apoenzymes the derivatives are split and that the activation is really due to the natural cofactor.[3, 11-12]

In contrast to their effects *in vitro*, some of these hydrazones of pyridoxal-5'-phosphate induce a strong inhibition of cerebral GAD when injected into animals and cause severe convulsions at the time GAD is inhibited.[3, 13, 14] In the case of L-glutamyl-γ-hydrazone, it has been postulated that this apparent contradiction between the effects *in vitro* and *in vivo* is probably due to the fact that *in vivo* the hydrazone inhibits the pyridoxal kinase (ATP-pyridoxal 5-phospho-transferase, EC 2.7.1.35) in brain and that the inhibition of GAD is secondary to this action, whereas *in vitro* the hydrazone is split in the presence of the GAD protein.[3]

To test further this hypothesis, several derivatives of pyridoxal-5'-phosphate were synthesized and their action *in vitro* and *in vivo* upon GAD and pyridoxal kinase activities in brain was compared with that of the reduced derivatives (substituted hydrazines). In the latter, the imino bond joining the cofactor with the hydrazine or hydrazide has been stabilized by the reduction.

MATERIALS AND METHODS

The L-glutamyl-γ-hydrazone, unsubstituted hydrazone, thiosemicarbazone and isonicotinylhydrazone of pyridoxal-5'-phosphate were prepared by the method of Sah[15] or by the method of McCormick and Snell.[16] The oximino derivative of amino-oxyacetic acid with pyridoxal phosphate (oxime-*O*-acetic acid) was synthesized in the same way.

Reduction of pyridoxal-5'-phosphate derivatives. In order to make the pyridoxal-5'-phosphate derivatives stable and in this way to avoid their possible rupture during the incubation with the enzymes, they were reduced by the addition of solid $NaBH_4$ to a suspension of the derivative in 0·05 M phosphate buffer, pH 7, or in water until the spectra of the compounds did not change any more with further addition of $NaBH_4$. In most cases the reducing agent had to be added in excess (about three times the amount of the derivative, by weight) in order to reduce all the compound present. It took 30–60 min to reduce it. In the course of the reduction, the suspensions of the derivatives were converted to solutions and their characteristic yellow color gradually disappeared. At the end of the reduction, the excess of borohydride was destroyed by the addition of acetic acid, until a pH of 5·5 to 6·0 was reached. The addition of $NaBH_4$ alone did not affect the activity of the enzymes studied. The L-glutamyl-γ-hydrazone was also reduced by hydrogenation at room temperature under 1 atmosphere of hydrogen until the uptake of the gas had ceased (about 2 hr), in the presence of platinum oxide as catalyst. The same phenomena of solubilization and decoloration were observed as with the reduction with $NaBH_4$. The spectrum and effects on the enzymes of the L-glutamyl-γ-hydrazone reduced by the two methods were identical.

The spectra of the unreduced and reduced derivatives were taken in a Cary recording spectrophotometer in phosphate buffer, pH 7·0. The purity of the reduced compounds was checked by paper chromatography in two different solvents (80% phenol and butanol–acetic acid–water, 4:2:1). They were visualized under an ultraviolet lamp and, when applicable, by spraying with ninhydrin. Although the R_f values of the unreduced derivatives in general did not differ very much from those of the reduced

ones, the color of the fluorescence was different and the contamination with unreduced derivative could be checked. In all the experiments, the derivatives were reduced immediately before the enzymatic assays or the administration to mice, since it was observed that their spectra changed considerably if stored, especially in dilute solutions.

Glutamate decarboxylase. In all experiments, Yale Swiss adult mice were used. GAD activity was determined by measuring the formation of $^{14}CO_2$ from L-glutamic acid-1-^{14}C (Volk; sp. act., 9 mc/m-mole) in incubation mixtures similar to those described by Roberts and Simonsen,[17] containing 0·2 μc of labeled glutamic acid and 0·033 M L-glutamic acid. The incubations were made in Warburg flasks in a final volume of 1·1 ml. For the experiments *in vitro*, the supernatants of brain homogenates (1:4, w/v) in Triton X-100 (0·5%, final concentration) centrifuged at 18,000 *g* for 30 min at 0–4° were used as the source of the enzyme (0·3 ml/flask), since it had been found that under these conditions GAD is activated by pyridoxal-5'-phosphate to a greater extent than under any other conditions, although the activity in the absence of the cofactor was less (unpublished observations). For the experiments *in vivo*, the complete water homogenate of brain from control mice or from mice treated with the derivatives was used as the source of the enzyme (0·3 ml/flask).

The $^{14}CO_2$ produced in the reaction was collected in 0·1 ml of hyamine hydroxide (1 M) in methanol, which was injected into small test tubes in the center well of the flasks at the end of the incubation, as described by Kravitz.[18] The incubation time was 30 min and the flasks were shaken for another 75 min after the hyamine injection and addition of 10% TCA from the side arm. The hyamine was transferred to scintillation counting vials containing 15 ml of scintillation fluid [4 g of 2,5-diphenyloxazole and 50 mg of 1,4-*bis*-2(5-phenyloxazolyl) benzene per liter of toluene] and the radioactivity was counted in a Nuclear-Chicago scintillation counter. The radioactivity of a blank flask with boiled enzyme was subtracted from all other values in all the experiments.

Pyridoxal kinase. For the determination of pyridoxal kinase activity, the incubation mixtures used were as described by McCormick *et al.*,[19] in a final volume of 2·4 ml. For the experiments *in vitro*, 0·3 mg of the enzyme purified from mice brain by the method of McCormick and Snell[20] until the third step [dialyzed fraction of precipitate in 60% $(NH_4)_2SO_4$] was used. The incubation time was 30 min at 37°. For the experiments *in vivo*, the supernatant obtained from centrifuging (18,500 *g*, 15 min) the homogenate (in 0·1 M phosphate buffer, pH 6·5; 1:4, w/v) of brains of control or treated mice was used as the source of the enzyme (0·4 ml/flask); in this case the incubation time was 60 min at 37°. The reaction was stopped by the addition of TCA.

After some preliminary experiments using the tyrosine decarboxylase method for measuring the pyridoxal phosphate formed in the reaction, it was found that a sufficient amount of the cofactor was produced to allow its determination with the phenylhydrazine reagent, as described by Wada and Snell,[21] using the total volume of incubation. More reproducible results were obtained with this method. The reaction was allowed to develop for exactly 30 min at 1° and the color was read in a Cary spectrophotometer at 410 mμ. In the experiments *in vitro*, the small amount of protein present did not interfere with the reading. However, in the experiments *in vivo*, the protein was centrifuged and the supernatant was used for the colorimetric procedure. The color produced by the pyridoxal present in the incubation mixture was corrected in all cases by using a blank with the enzyme boiled or without enzyme. Under the

experimental conditions described, the amount of pyridoxal-5'-phosphate formed was proportional to the amount of protein present in the incubation mixture.

In the experiments *in vitro*, it was found that some of the pyridoxal phosphate hydrazones studied were converted to the phenylhydrazone of pyridoxal-5'-phosphate when the phenylhydrazine reagent was added. However, at the concentrations of the compounds employed, appropriate blanks without enzyme were used to correct the results. The substituted hydrazines, as expected, did not give color with the phenylhydrazine reagent. This was also the case with the unreduced oxime-*O*-acetic acid. In the experiments *in vitro*, a blank with boiled enzyme was used for each mouse in order to correct the results for the small amount of pyridoxal-5'-phosphate present in the tissue. The protein in the purified pyridoxal kinase was measured by spectrophotometric methods.[22]

RESULTS

Although the spectra of the unreduced compounds were different from one another, the spectra of all the reduced derivatives showed a maximum at 325 mμ. This peak corresponds to the free pyridoxamine phosphate,[23] as was to be expected for a compound with a C—N bond in position 4 of the pyridoxal-5'-phosphate molecule. This maximum appears also when the coenzyme linked to proteins is reduced.[24]

Glutamate decarboxylase. The four hydrazones tested activated brain GAD when added *in vitro*, although not to the same extent as pyridoxal-5'-phosphate. The isonicotinylhydrazone was the most potent derivative and the thiosemicarbazone the least potent (Table 1). In contrast to these results, the four substituted hydrazines had no effect on GAD activity when added at the same concentration. The pyridoxal-5'-phosphate oxime-*O*-acetic acid, on the other hand, inhibited GAD activity about 50 per cent both in the unreduced and reduced forms (Table 1). The addition of pyridoxal-5'-phosphate ($0{\cdot}92 \times 10^{-4}$ M) did not reverse the inhibition produced by the unreduced oxime-*O*-acetic acid. Under these conditions the mean value of three determinations was 5199 cpm, which indicates an inhibition of 55·7 per cent when compared to the 11,735 control value of Table 1.

TABLE 1. EFFECTS *IN VITRO* OF PYRIDOXAL-5'-PHOSPHATE AND OF UNREDUCED AND REDUCED PYRIDOXAL-5'-PHOSPHATE DERIVATIVES ON BRAIN GAD ACTIVITY

Derivative*	GAD activity†	% Change
None (24)	6076 [5408–7136]	
Pyridoxal-5'-phosphate (18)	11,735 [10,474–13,348]	+93·1
L-Glutamyl-γ-hydrazone (4)	10,533 [10,327–10,976]	+73·4
Reduced (7)	6473 [5552–7124]	+6·5
Hydrazone (4)	10,080 [9512–11,138)	+65·9
Reduced (4)	6368 [5725–7478]	+4·8
Isonicotinoylhydrazone (8)	11,023 [10,029–12,165]	+81·4
Reduced (7)	6253 [5357–7070]	+2·7
Thiosemicarbazone (5)	9552 [9168–10,079]	+57·2
Reduced (6)	6125 [5370–6609]	+0·8
Oxime-*O*-acetic acid (9)	3122 [2804–3374]	−48·6
Reduced (5)	3002 [2807–3288]	−50·6

* The final concentration of pyridoxal-5'-phosphate or its derivatives was $0{\cdot}92 \times 10^{-4}$ M.

† The results are expressed as cpm in $^{14}CO_2$ produced in 30 min of incubation under the conditions indicated in Methods (mean values). Number of determinations is in parentheses; maximal and minimal values are in brackets.

When the hydrazone or the L-glutamyl-γ-hydrazone was injected into mice at the doses indicated in Table 2, they produced fatal convulsions in 30–45 min. The brain GAD activity of mice decapitated when they were about to die in convulsions was considerably inhibited. The addition of pyridoxal-5′-phosphate to the incubation mixtures completely reversed the inhibition (Table 2). The isonicotinylhydrazone, the thiosemicarbazone and the oxime-*O*-acetic acid, on the contrary, did not cause any apparent behavioral alterations in the mice, nor did they alter the brain GAD activity when injected into mice at equimolar doses (Table 2).

TABLE 2. EFFECTS *IN VIVO* OF UNREDUCED AND REDUCED PYRIDOXAL-5′-PHOSPHATE DERIVATIVES ON BRAIN GAD ACTIVITY IN THE ABSENCE AND IN THE PRESENCE OF PYRIDOXAL-5′-PHOSPHATE

Derivative*	No pyridoxal-5′-phosphate added	% Inhibition	Pyridoxal-5′-phosphate added	% Inhibition	Convulsions†
Control (injected with 0·9% NaCl)	10,197 [9,210–11,203]		14,409 [12,792–15,412]		
L-Glutamyl-γ-hydrazone (205 μmoles/kg)	5888‡	42·3	13,807‡	4·2	yes
Reduced	11,234	0	15,142	0	no
Hydrazone (220 μmoles/kg)	5273	48·3	13,432	6·8	yes
Reduced	9446	7·4	14,940	0	no
Isonicotinoylhydrazone (220 μmole/kg)	8928	12·4	13,764	4·5	no
Reduced	10,484	0	14,731	0	no
Thiosemicarbazone (220 μmoles/kg)	10,127	0·7	14,628	0	no
Reduced	10,259	0	14,046	2·5	no
Oxime-*O*-acetic acid (220 μmole/kg)	9527	6·6	14,461	0	no

* The derivatives were injected i.p. at the doses indicated. The mice which did not convulse were decapitated at 45–60 min after treatment. GAD activity is expressed as cpm in $^{14}CO_2$ produced in 30 min of incubation under the conditions indicated in Methods. The mean value of 8 control mice (range in brackets) is shown. In the treated mice, the values are the mean of 2 animals, which did not differ by more than 12 per cent. Pyridoxal-5′-phosphate concentration, when added, was $0{\cdot}92 \times 10^{-4}$ M.

† Six to 12 mice were injected with each derivative to confirm the presence or absence of convulsant effect.

‡ Value of only 1 animal (see ref. 3).

Interestingly, none of the reduced derivatives caused convulsions or modified brain GAD activity when injected at doses equimolar to those of the unreduced compounds.

Pyridoxal kinase. At the concentrations indicated in Table 3, the hydrazone, the L-glutamyl-γ-hydrazone and the isonicotinylhydrazone produced a slight but significant inhibition of brain pyridoxal kinase. With the exception of the substituted hydrazine corresponding to L-glutamyl-γ-hydrazone, which had a slight inhibitory effect, none of the reduced derivatives studied modified the activity of the kinase (Table 3.).

The effects of the derivatives on brain pyridoxal kinase *in vivo* were parallel to their action on GAD activity. The two convulsant compounds, hydrazone and L-gluta-

myl-γ-hydrazone, inhibited it, while the other two hydrazones had no effect (Table 4). The reduced derivatives tested, as in the case of GAD, failed to affect the activity of pyridoxal kinase when injected at doses equimolar to those of the hydrazones (Table 4).

TABLE 3. EFFECTS *IN VITRO* OF UNREDUCED AND REDUCED PYRIDOXAL-5'-PHOSPHATE DERIVATIVES ON BRAIN PYRIDOXAL KINASE ACTIVITY

Derivative	Pyridoxal kinase activity*	% Inhibition	Significance (*t*-test)
None (11)	11·73 ± 0·30		
L-glutamyl-γ-hydrazone (6) (10^{-5} M)	10·25 ± 0·26	12·6	P<0·01
Reduced (9)	12·01 ± 0·33	0	
Hydrazone (4) (10^{-5} M)	9·08 ± 0·61	22·6	P<0·01
Reduced (3)	12·20 ± 0·15	0	
Isonicotinoylhydrazone (4) (5×10^{-5} M)	10·50 ± 0·27	10·5	P < 0·01
Reduced (4)	11·88 ± 0·70	0	
None (13)†	7·52 ± 0·07		
Thiosemicarbazone (7) (5×10^{-5} M)	7·71 ± 0·24	0	
Reduced (5)	7·58 ± 0·21	0	

* The results are expressed in micrograms of pyridoxal-5'-phosphate produced in 30 min of incubation under the conditions indicated in Methods. Mean ± S.E.M. Number of determinations is in parentheses.

† Different preparation of the enzyme, with lower specific activity.

TABLE 4. EFFECTS *IN VIVO* OF UNREDUCED AND REDUCED PYRIDOXAL-5'-PHOSPHATE DERIVATIVES ON BRAIN PYRIDOXAL KINASE ACTIVITY

Derivative*	Pyridoxal kinase activity†	% Inhibition	Significance (*t*-test)
Control (19)	8·02 ± 0·18		
L-Glutamyl-γ-hydrazone (7)	5·13 ± 0·31	36·0	P < 0·001
Reduced (7)	7·30 ± 0·23	9·0	
Hydrazone (7)	5·49 ± 0·43	31·5	P < 0·001
Reduced (4)	8·05 ± 0·26	0	
Isonicotinoylhydrazone (4)	8·25 ± 0·26	0	
Thiosemicarbazone (4)	8·15 ± 0·13	0	

* See Table 2 for doses and other related data.

† The results are expressed in micrograms of pyridoxal-5'-phosphate produced in 1 hr of incubation under the conditions indicated in Methods. Mean ± S.E.M. Number of animals is in parentheses.

DISCUSSION

The results of the experiments with GAD *in vitro* are in agreement with the previously reported observation that hydrazones of pyridoxal-5'-phosphate act in a manner similar to pyridoxal-5'-phosphate itself with this enzyme.[3, 5, 9] One explanation of this fact is that hydrazones are sufficiently unstable to yield pyridoxal-5'-

phosphate, which then reacts with the apoenzyme.[3, 11, 12] Others argue that the hydrazones themselves possess coenzymatic activity.[4–6] That this is not probable is shown by our negative results with the corresponding substituted hydrazines. Reduction of the hydrazones destroys the potential aldehyde function and, according to present theories, this group is essential for catalysis. One can reasonably conclude that pyridoxal-5′-phosphate released from the hydrazones is responsible for the observed activation of GAD. How the pyridoxal phosphate is liberated remains an open question. Transaldimination with some reactive NH_2 groups of the enzyme is a distinct possibility already proposed by Torchinsky[11] for aspartate aminotransferase.

The fact that oxime-*O*-acetic acid inhibits GAD activity *in vitro* can be explained by the greater stability of this compound in comparison with that of the hydrazones. This can be inferred from the finding that aminooxyacetic acid was not displaced from the pyridoxal-5′-phosphate molecule by the phenylhydrazine reagent, as occurred with the hydrazones (see Methods). Thus, pyridoxal phosphate could not be liberated, as is probably the case with the hydrazones, and the inhibition of GAD by the oxime could conceivably result from a firm attachment of the oxime on the active site of the enzyme. The fact that the reduced oxime also inhibited GAD activity (Table 1) and that the addition of pyridoxal-5′-phosphate did not reverse the inhibition produced by the unreduced compound (see text, in Results) support this idea.

The slight inhibition of pyridoxal kinase *in vitro* by some of the hydrazones studied (Table 3) does not agree with the negative results of McCormick and Snell.[16] The concentrations of the substances used in their work are not indicated and therefore it is difficult to compare the different results obtained.

The results of the experiments *in vivo* provide additional evidence that inhibition of GAD activity leads to convulsions.[25–27] Only the hydrazone and the L-glutamyl-γ-hydrazone produced convulsions and these two derivatives were the strongest inhibitors of brain GAD (48 and 42 per cent inhibition respectively). The next in line is the isonicotinylhydrazone, which inhibited GAD activity only 12 per cent and had no convulsant action. It is noteworthy that oxime-*O*-acetic acid, which inhibited GAD *in vitro*, was not a convulsant and did not inhibit GAD *in vivo*. This result agrees with the previously reported lack of effect of the simultaneous administration of pyridoxal-5′-phosphate and aminooxyacetic acid, while the simultaneous injection of L-glutamic acid-γ-hydrazide and pyridoxal-5′-phosphate produces convulsions and inhibits GAD activity.[3]

The experiments *in vivo* also indicate that the inhibition of GAD produced by the two convulsant derivatives is probably secondary to their inhibitory effect on pyridoxal kinase. This conclusion is based on the fact that only the compounds which inhibited GAD activity showed any effect on pyridoxal kinase (Tables 2 and 4). The reversal of the GAD inhibition by the addition of pyridoxal-5′-phosphate (Table 2) supports this conclusion. The inhibition of pyridoxal kinase would result in a decrease of the endogenous pyridoxal phosphate concentration and, as a consequence in an inhibition of GAD activity. In this regard, it is pertinent to mention a paper by Minard[28] in which he found an excellent correlation between the concentration of pyridoxal-5′-phosphate and the activity of GAD in brain. It would be desirable to know if the inhibition of pyridoxal kinase observed after treatment with the hydrazone or the L-glutamyl-γ-hydrazone results in a decrease in the level of the cofactor in brain great enough to account for the inhibition of GAD observed under these conditions.

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