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3-ISOXAZOLIDONE—AN INHIBITOR OF GABA METABOLISM

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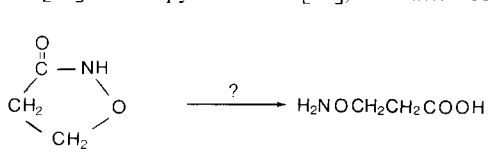
Abstract—3-Isoxazolidone, a cyclic analogue of aminooxypropionic acid, was prepared and evaluated for its effect on γ-aminobutyric acid (GABA) metabolism. Its administration to animals resulted in potent inhibitions of brain glutamic acid decarboxylase (GAD) and GABA-α-oxoglutarate aminotransferase (GABA-T) activities. A small but significant increase in brain GABA content was observed. The inhibiting effects varied with the experimental species being greatest in chicks and least in mice. The enzyme inhibitions were not prevented by the concurrent administration of pyridoxine to the animals nor by the addition of pyridoxal phosphate to the assay system. Additions of 3-isoxazolidone directly to brain homogenates inhibited the activities of both GAD and GABA-T but not to the extent that might be expected from the studies *in vivo*.

There is now convincing evidence that γ-aminobutyric acid (GABA) is an inhibitory neurotransmitter substance in the central nervous system [1, 2], where it is formed by the action of glutamic acid decarboxylase (GAD) [3, 4] and is degraded by the action of GABA-α-oxoglutarate aminotransferase (GABA-T) [5, 6]. Not surprisingly, the possibility that a drug-induced elevation in GABA content might accentuate the inhibitory functions within the brain has stimulated a vigorous search for agents which inhibit GABA-T activity specifically. Unfortunately, potent GABA-T inhibitors, such as hydrazine, hydrazides, hydrazinopropionic acid and hydroxylamine, also inhibit the GABA-synthesizing enzyme GAD [7–10]. Even the most commonly used GABA-T inhibitor, aminooxyacetic acid, significantly inhibits GAD activity for several hr after its administration [11]. Aminooxypropionic acid, a close structural analogue of aminooxyacetic acid, is a more potent inhibitor of GABA-T activity *in vitro* than is aminooxyacetic acid but unfortunately it does not inhibit the enzyme system *in vivo* [12] due, presumably, to its inability to cross the blood brain barrier.

A procedure which is frequently used to circumvent the impermeability of the blood–brain barrier is the administration of an analogue which is less polar then the compound in question, and which penetrates into the brain, where it is converted enzymatically to the desired active compound. Examples of this procedure which have been used with respect to GABA-T inhibitors include unsubstituted and substituted pyrrolidones [13] and 3-pyrazolidone [14], the latter compound hydrolyzing to the potent inhibitor hydrazinopropionic acid.



3-isoxazolidone aminooxypropionic acid

Fig. 1. Proposed metabolic transformation of 3-isoxazolidone.

In view of the above situation, we attempted to circumvent the impermeability of the blood–brain barrier to aminooxypropionic acid by administering its close structural analogue, 3-isoxazolidone, on the premise that brain tissue enzymes might hydrolyze the drug, resulting in the formation of aminooxypropionic acid itself (Fig. 1). The present report documents the effect of 3-isoxazolidone on GABA metabolism under conditions both *in vivo* and *in vitro*.

MATERIALS AND METHODS

Synthesis of 3-isoxazolidone

3-Isoxazolidone was prepared by the method of Shunk *et al.* [14]. Commercially available β-chloropropionyl chloride (Aldrich)(25 g) was treated with hydroxylamine hydrochloride and sodium hydroxide at 0°. Subsequent extraction of the reaction mixture with butanol and evaporation to dryness gave a solid which upon extraction with several portions of ether yielded β-chloropropionohydroxamic acid (9.0 g). The hydroxamic acid (3.0 g) was warmed to 50° (20 min) with sodium hydroxide (two equivalents), the resulting mixture neutralized with hydrochloric acid, evaporated to dryness and subsequently extracted with ethanol. Evaporation of the solvent gave a solid residue which when triturated with boiling ether gave the desired 3-isoxazolidone (1.45 g) m.p. 68–71°, lit. 68–70°.

Animals

Male Swiss albino mice (25 g), male Wistar rats (200 g) and white Leghorn cockerels (150–220 g) were used in the experiments. All animals were fasted for 24 hr prior to use.

Drug administration

Solutions of 3-isoxazolidone were prepared daily in 0.9% (w/v) NaCl, the pH being adjusted to 7.0 immediately before use. The final concentration of in-

hibitor was adjusted so that the required dosage was administered to mice in a volume equivalent to 0.5 per cent of the body weight, and to rats and chicks in a volume equivalent to 1.0 per cent of the body weight. When animals were treated with 3-isoxazolidone and pyridoxine, in eqimolar proportions, both compounds were dissolved in the same solution. All injections were intramuscular and the injected animals were kept in a laboratory with minimal background noise.

Preparation of brain homogenates

The animals were decapitated so that the heads fell directly into liquid nitrogen. The heads were stored in the liquid nitrogen until required for the assays. The heads were removed from the liquid nitrogen and partially thawed by placing them in cold 0.9% NaCl for 30 min. The whole brain was removed and weighed, and homogenates were prepared in a Potter–Elvehjem homogenizer using the media described below for the specific assay.

Biochemical analysis

Brain GABA levels. The brains were excised, homogenized in water, and GABA-containing extracts prepared as described previously [15]. The GABA content of the extracts was determined enzymically as described by Jakoby [16], except that 0.15 ml of 1 M Na_2SO_4/tube was added. The total volume was 1.2 ml.

Enzyme activities. Glutamic acid decarboxylase activity was determined by trapping $^{14}CO_2$ liberated from [1-^{14}C]DL-glutamic acid (Amersham/Searle Corp.); sp. act. was 29 mCi/m-mole using the procedure of Roberts and Simonsen [17]. The contents of each incubation flask consisted of 0.4 ml buffer substrate and 0.4 ml of 10% (w/v) brain homogenate prepared in water containing 0.25% (w/v) Triton X-100 and 0.02 M mercaptoethanol. Incubation was carried out under nitrogen at 37° for 10 min. The reaction was terminated by the addition of 0.1 ml of 8 N H_2SO_4. The $^{14}CO_2$ liberated was trapped in 1.0 M hyamine hydroxide (in methanol) and the radioactivity counted in an Isocap 300 liquid scintillation system. Unless otherwise stated, pyridoxal phosphate was not added to the incubation medium. When included in the medium, its final concentration was 0.002 M.

The activity of GABA-T in brain homogenates was monitored by measuring the rate of succinic semialdehyde formation. Homogenates were prepared as described above, except that the concentration of Triton X-100 was 1.25% (w/v), and the GABA-T activity was determined by the method of Salvador and Albers [18] as modified by Salganicoff and De Robertis [19]. A portion of the homogenate (0.2 ml) was added to 5.0 ml of a buffer solution (pH 8.4) containing 0.1 M α-oxoglutarate and 0.25 M GABA. Incubation was carried out for 60 min at 38°. Samples (0.5 ml) were removed from the incubation mixture at zero time and at 60 min for the fluorometric determination of succinic semialdehyde content using 0.25 M 3,5-diaminobenzoic acid.

The effect *in vitro* of 3-isoxazolidone on the enzyme activities was studied by adding it, dissolved in buffer appropriate to the enzyme assay, directly to the incubation vessels. The final concentration of 3-isoxazolidone in the incubation medium is given in the pertinent tables.

Determination of CT_{50} values

This value, expressed in min, was the time required after administration of convulsant agent for seizures to occur in 50 per cent of the animals. The CT_{50} value was determined by plotting cumulative percentage convulsed as a function of time on logarithmic probit paper as described by Paton [20].

Statistical treatment

When required, statistical analysis was carried out using analysis of variance and Duncan's multiple range test.

RESULTS

Effect in vivo *of 3-isoxazolidone on GABA metabolism*

The intramuscular administration of 3-isoxazolidone to mice at concentrations of 1, 1.5 and 2.0 m-moles/kg produced a marked inhibitory action on both the decarboxylation of glutamic acid and the transamination of GABA (Table 1). However, the effect on GABA-T was much more pronounced than that on GAD, resulting in a net elevation in GABA level. The decrease in the activities of both enzymes and the increase in GABA concentration were all dose dependent. The 3-isoxazolidone-induced inhibitions in the activities of both GAD and GABA-T were relatively slow in developing, maximum inhibitions occurring 3 hr after administration of the drug (Fig. 2). Similarly, the return to normal activities was also slow, and significant inhibitions in both GAD and GABA-T activities still existed 24 hr after administration of the 3-isoxazolidone.

The concurrent administration of pyridoxine with 3-isoxazolidone did not alter the isoxazolidone-induced changes in GAD activity and GABA-T acti-

Table 1. Effect of the administration of different amounts of 3-isoxazolidone on the brain GABA content and metabolism in mice*

3-Isoxazolidone (m-moles/kg)	GAD (μmoles/g/hr)	GAD Inhibition (%)	GABA-T (μmoles/g/hr)	GABA-T Inhibition (%)	GABA (μmoles/g)
Control	37.3 ± 2.4		191 ± 3		2.05 ± 0.05
1.0	29.1 ± 0.6†	22	107 ± 4†	44	2.31 ± 0.08
1.5	25.0 ± 1.4†	33	68 ± 4†	64	2.43 ± 0.04
2.0	18.2 ± 0.8†	51	41 ± 5†	79	3.40 ± 0.28†

* Mice were killed 6 hr after the administration of 3-isoxazolidone. All values are the mean ± S. E. M. for four samples each containing three brains for GABA determinations and one brain for measurement of the enzyme activities.

† Significantly different from control $P < 0.01$

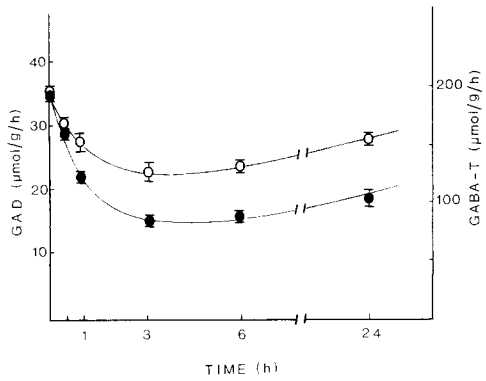


Fig. 2. Effect of 3-isoxazolidone (1.5 m-moles/kg) on GAD and GABA-T activities in mouse brain. Key: (○——○) GAD activity; (●- - -●) GABA-T activity. Each value is the mean ± S.E.M. for four samples.

vity, nor did it alter significantly the net change in GABA concentration (Table 2). Pyridoxine treatment *per se* lowered the GABA content of brain although it was without effect on the enzyme activities. Addition of the cofactor pyridoxal phosphate (PALP) to the incubation medium brought about the anticipated increase in GAD activity but was without effect on the degree of inhibition caused by the administration of the 3-isoxazolidone to the mice (Table 2). In contrast, added PALP *per se* greatly decreased GABA-T activity but was again ineffective in ameliorating the 3-isoxazolidone-induced inhibition.

The administration of 3-isoxazolidone had a more marked inhibitory effect on brain GAD and GABA-T activities in rats and chicks than in mice, the drug being a particularly potent inhibitor in the chicks (Table 3). The sensitivity to isoxazolidone of the GAD enzyme system relative to that of the GABA-T enzyme system was, however, similar in all three species.

Effect in vitro *of 3-isoxazolidone on GABA metabolism*

3-Isoxazolidone was a rather poor inhibitor of GABA metabolism under conditions *in vitro* (Table 4). At a concentration of 10^{-3} M it inhibited GAD activity by only 24 per cent and GABA-T activity not at all. Even at 10^{-2} M isoxazolidone inhibited the GABA-T enzyme system by a mere 28 per cent.

Effect of 3-isoxazolidone on isonicotinic acid hydrazide-induced seizures

Since 3-isoxazolidone causes a small but significant elevation in brain GABA content (Table 1), the efficacy of the drug as an anticonvulsant agent was examined. The prior administration of isoxazolidone (1.5 m-moles/kg) did not, however, significantly delay the onset of the isonicotinic acid hydrazide (INH)-induced seizures (Fig. 3). Moreover, 3-isoxazolidone *per se* induced seizures in mice (CT_{50}, 107 min) when administered at a dosage level of 3 m-moles/kg (Fig. 3).

DISCUSSION

The data presented here indicate that 3-isoxazolidone, or a metabolite thereof, penetrates the blood–brain barrier and inhibits both GAD and GABA-T activities in the brain. Whether these inhibitions are due to the action of the drug *per se* or whether they are caused by aminooxypropionic acid formed *in vivo*

Table 2. Effect of the administration of 3-isoxazolidone on GABA metabolism in mouse brain

Expt. No.	Treatment* (m-moles/kg)	GABA (μmoles/g)	GABA-T (μmoles/g/hr) No PALP	GABA-T (μmoles/g/hr) PALP†	GAD (μmoles/g/hr) No PALP	GAD (μmoles/g/hr) PALP†	Inhibition (%) No PALP	Inhibition (%) PALP
1	Control	2.07 ± 0.04	201 ± 1		37.9 ± 1.4	61.6 ± 0.7		
	3-Isoxazolidone	2.58 ± 0.15‡	86 ± 3‡		28.8 ± 1.3‡	44.2 ± 0.7‡	24	28
	Pyridoxine	1.47 ± 0.04‡	201 ± 3		36.3 ± 3.2	63.8 ± 3.6		
	3-Isoxazolidone + Pyridoxine	1.75 ± 0.10	87 ± 6‡		26.8 ± 1.8‡	46.9 ± 2.7‡	29	24
2	Control		185 ± 3	36 ± 1				
	3-Isoxazolidone		65 ± 2‡	8 ± 1‡				

* Mice were sacrificed 3 hr after administration of the drugs, which were given at a dosage level of 1.5 m-moles/kg for each drug.

† PALP was added to the incubation medium to give a final concentration of 0.002 M.

‡ Significantly different from control values, $P < 0.01$. All values were the mean ± S. E. M. for four samples each containing three brains for the GABA estimation and one brain for the determination of the enzyme activities, except for control values, which are the mean for eight samples.

Table 3. Effect of 3-isoxazolidone on GAD and GABA-T activities in different species*

Species	Treatment (m-moles/kg)	GAD (μmoles/g/hr) Control	GAD (μmoles/g/hr) Test	Inhibition (%)	GABA-T (μmoles/g/hr) Control	GABA-T (μmoles/g/hr) Test	Inhibition (%)
Mouse	1.5	36.2 ± 0.5	23.1 ± 1.5	36	196.5 ± 1.4	84.1 ± 5.9	59
Rat	1.5	29.9 ± 1.4	10.3 ± 1.2	66	173.2 ± 0.9	24.3 ± 2.6	86
Chick	0.5	28.4 ± 1.4	16.7 ± 1.5	42	154.7 ± 3.7	33.5 ± 2.5	78
	1.5	27.3 ± 1.2	9.3 ± 1.3	66	152.7 ± 2.9	6.6 ± 0.8	98

* Animals were killed 3 hr after the administration of 3-isoxazolidone. All values are the mean ± S. E. M. for four brains.

Table 4. Effect of 3-isoxazolidone on GAD and GABA-T activities *in vitro**

Species	3-Isoxazolidone (M)†	GAD (μmoles/g/hr)	GAD Inhibition (%)	GABA-T (μmoles/g/hr)	GABA-T Inhibition (%)
Mouse	None	35.4 ± 1.4		197 ± 3	
	1.0×10^{-3}	27.0 ± 2.0‡	24	195 ± 3	1
	1.0×10^{-2}	3.6 ± 1.1‡	90	142 ± 3‡	28
Chick	None	31.0 ± 0.7			
	1.0×10^{-3}	23.2 ± 1.6‡	25		
	1.0×10^{-2}	2.0 ± 0.5‡	94		

* All values are the mean ± S. E. M. for four brains.
† Final concentration in the incubation medium.
‡ Significantly different from control ($P < 0.01$).

from the 3-isoxazolidone is not clear. Although the data show that 3-isoxazolidone is a potent inhibitor of GABA-T and GAD activities, being superior to isonicotinic acid hydrazide in this respect [21], its inhibitory action on GABA-T activity in mice is much less than that of aminooxyacetic acid [11]. In addition, the ratio GABA-T inhibition/GAD inhibition observed with 3-isoxazolidone is closer to unity than that obtained with aminooxyacetic acid [11], thereby suggesting that the compound is inhibiting by virtue of its carbonyl-trapping properties (both GAD and GABA-T are pyridoxal phosphate enzymes), rather than as a result of it being a structural analogue of the GABA-T substrate, GABA. This finding tends to eliminate the possibility of the inhibition being due to aminooxypropionic acid formed from the 3-isoxazolidone. On the other hand, in contrast to the situation with hydrazides [22], the addition of pyridoxal phosphate to the assay system did not prevent the 3-isoxazolidone-induced inhibition of GAD. Likewise, in contrast to the situation with hydrazides [23], the simultaneous administration of pyridoxine did not protect against the 3-isoxazolidone-induced inhibition of GAD (Table 2). These findings, therefore, argue against a mechanism of inhibition based simply on the carbonyl-trapping properties of 3-isoxazolidone.

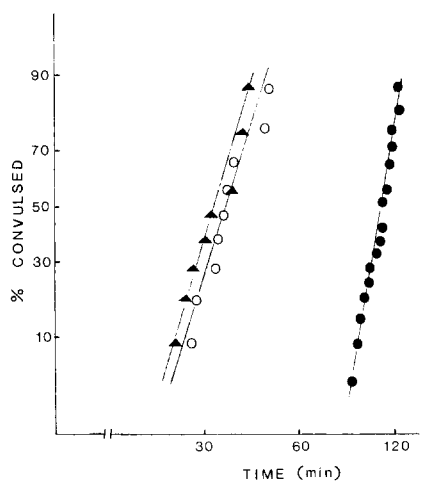


Fig. 3. Effect of 3-isoxazolidone on isonicotinic acid hydrazide (INH)-induced seizures in mice. Key: (▲——▲) 2.2 m-moles INH/kg; (○——○) 2.2 m-moles INH/kg + 1.5 m-moles 3-isoxazolidone/kg; (●——●) 3.0 m-moles 3-isoxazolidone/kg. Each group consisted of twenty mice. The data are presented as a logarithmic probit plot.

The time required after the administration of 3-isoxazolidone for maximum inhibition of GAD to develop (Fig. 1) is much longer than that required for maximum inhibition after treatment with aminooxyacetic acid or hydrazides [11, 24]. This could be due to a slower penetration of 3-isoxazolidone into the brain. On the other hand it might indicate that 3-isoxazolidone has to be converted to some other derivative which is the active inhibitor. Similarly, the comparably greater inhibition of 3-isoxazolidone in chicks and rats than in mice could be explained by either the blood–brain barrier in the former two species being more permeable to the compound or, alternately, by the presence of a more active enzyme system converting the 3-isoxazolidone to the active inhibitor. However, the fact that 3-isoxazolidone is an equally effective inhibitor *in vitro* of GAD activity in homogenates from chicks and mice (Table 4) favors the blood–brain barrier hypothesis since the conversion enzyme might be expected to be present in the brain homogenate, although of course the assay condition may not have been favorable for its activity.

An interesting result obtained in the present study is the significant decrease in the concentration of GABA in the brains of mice treated with pyridoxine. A similar effect was observed previously with chicks [24]. The reason for this decrease is unclear but one possiblity is a change in the rate of membrane transport with respect to GABA, thereby altering the accessibility of the amino acid to its degradative enzyme. Stimulation of amino acid transport by B_6 vitamins has been reported previously although the mode of action is probably indirect [25].

In summary, a drug has been developed which is a potent inhibitor of both the GABA-synthesizing and the GABA-degrading enzyme systems but the mechanisms involved in the inhibitions remain uncertain. In view of the possible presence in brain homogenates of an enzyme which converts 3-isoxazolidone to its active inhibitory compound, purification of GAD and GABA-T is a necessary prerequisite before kinetic studies with 3-isoxazolidone can be carried out. Preliminary work on the purification of these enzymes is now under way with a view to pursuing this experimental approach toward a resolution of the above uncertainties. Although 3-isoxazolidone may prove useful as a tool with which to study GAD and GABA-T enzyme systems, its potent inhibition of GAD activity with the attendant danger of seizures [26] precludes any role for the compound as a possible therapeutic agent.

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REFERENCES

1. D. R. Curtis and G. A. R. Johnston, in *Handbook of Neurochemistry* (Ed. A. Lajtha), Vol. 4, pp. 115. Plenum Press, New York (1970).
2. K. Krnjevic, *Nature, Lond.* **228**, 119 (1970).
3. E. Roberts and S. Frankel, *J. biol. Chem.* **187**, 55 (1950).
4. E. Roberts and S. Frankel, *J. biol. Chem.* **188**, 789 (1951).
5. S. P. Bessman, J. Rossen and E. C. Layne, *J. biol. Chem.* **201**, 385 (1953).
6. E. Roberts and H. M. Bregoff, *J. biol. Chem.* **201**, 393 (1953).
7. M. A. Medina, *J. Pharmac. exp. Ther.* **140**, 133 (1963).
8. J. D. Wood, D. K. J. Gorecki, J. R. Dimmock and E. M. Hawes, *Can. J. Physiol. Pharmac.* **53**, 47 (1975).
9. J. D. Wood and S. J. Peesker, *J. Neurochem.* **25**, 277 (1975).
10. N. M. Van Gelder, *J. Neurochem.* **15**, 747 (1968).
11. J. D. Wood and S. J. Peesker, *J. Neurochem.* **20**, 379 (1973).
12. E. J. Schumann, L. A. Paquette, R. V. Heinzelman, D. P. Wallach, J. P. Da Vanzo and M. E. Greig, *J. mednl. pharm. Chem.* **5**, 464 (1962).
13. G. Carvajal, M. Russek, R. Tapia and G. Massieu, *Biochem. Pharmac.* **13**, 1059 (1964).
14. C. H. Shunk, F. W. Bachelor and K. Folkers, *J. org. Chem.* **22**, 76 (1957).
15. D. E. Abrahams and J. D. Wood, *J. Neurochem.* **17**, 1197 (1970).
16. B. Jakoby, in *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), Vol. V, p. 765. Academic Press, New York (1962).
17. E. Roberts and D. G. Simonsen, *Biochem. Pharmac.* **12**, 113 (1963).
18. A. Salvador and R. Albers, *J. biol. Chem.* **234**, 922 (1959).
19. L. Salganicoff and E. De Robertis, *J. Neurochem.* **12**, 287 (1965).
20. W. D. M. Paton, *Br. J. Pharmac. Chemother.* **29**, 350 (1967).
21. R. E. Casey and J. D. Wood, *Comp. Biochem. Physiol.* **45B**, 741 (1973).
22. K. F. Killam and J. A. Bain, *J. Pharmac. exp. Ther.* **119**, 255 (1957).
23. H. Balzer, P. Holtz and D. Palm, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **239**, 520 (1960).
24. J. D. Wood and S. J. Peesker, *J. Neurochem.* **19**, 1527 (1972).
25. H. N. Christensen, *Adv. Biochem. Psychopharmac.* **4**, 39 (1972).
26. J. D. Wood and S. J. Peesker, *J. Neurochem.* **23**, 703 (1974).