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# Molecular Basis for the Irreversible Inhibition of 4-Aminobutyric Acid:2-Oxoglutarate and L-Ornithine:2-Oxoacid Aminotransferases by 3-Amino-1,5-Cyclohexadienyl Carboxylic Acid (Isogabaculine)

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SUMMARY

METCALF, B. W. AND M. J. JUNG. Molecular basis for the irreversible inhibition of 4-aminobutyric acid:2-oxoglutarate and L-ornithine:2-oxoacid aminotransferases by 3-amino-1,5-cyclohexadienyl carboxylic acid (isogabaculine). *Mol. Pharmacol.* **16:** 539–545 (1979).

Isogabaculine reacts with pyridoxal phosphate to form N-meta-carboxyphenylpyridoxamine phosphate as does its natural isomer gabaculine. The two compounds are almost equipotent *in vitro* and *in vivo* in inhibiting 4-amino-butyrate (GABA) aminotransferase and L-ornithine aminotransferase and in elevating brain GABA levels.

## INTRODUCTION

In recent years the search for enzyme-activated irreversible inhibitors of aminotransferases and especially of 4-aminobutyrate:2-oxoglutarate aminotransferase (E.C. 2.6.1.19; GABA-T)[1] has been actively pursued (1) because of the pharmacological potential of such compounds (2). The known inhibitors of GABA-T can be classified in three types. An electrophilic species can be generated in the enzyme active site by enzyme-catalyzed elimination of a substituent in $\beta$ position to the amine function of the GABA analogue. An example is ethanolamine-O-sulfate, the first enzyme-activated, irreversible inhibitor of GABA-T described (3). Alternatively, the electrophilic species can be generated by the normal enzyme-catalyzed prototropy as for 4-aminohex-5-enoic acid (4) or by an abnormal prototropy as has been postulated for 4-aminohex-5-ynoic acid (5). Finally, gabaculine inhibits GABA-T by forming a stable adduct with the pyridoxal-5'-phosphate present in the active site (6). In that specific case the adduct, meta-carboxyphenyl pyridoxamine phosphate has a high affinity for the active site and does not dissociate under nondenaturing conditions (6). This last type of inhibitor could be especially useful because the quasi-irreversible inhibition does not rely on the reaction of a nucleophilic group of the enzyme's active site with the activated inhibitor. Gabaculine is the most potent inhibitor of GABA-T both *in vitro* (6) and *in vivo* (7). We decided, therefore, to investigate whether the double-bond isomers of gabaculine would also in-

[1] The abbreviations used are: GABA, 4-aminobutyric acid; GABA-T, 4-amino-butyrate:2-oxoglutarate aminotransferase; gabaculine, 5-amino-1,3-cyclohexadienyl carboxylic acid; Orn-T, ornithine transferase (L-ornithine:2-oxoacid aminotransferase); GAD, glutamate decarboxylase (glutamate-1-carboxylyase).



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hibit GABA-T by aromatization. We report here that 3-amino-1,5-cyclohexadienyl carboxylic acid does in fact react with pyridoxal-phosphate in the same way that gabaculine does and that its potency and selectivity toward pyridoxal-phosphate dependent enzymes are very similar to those of gabaculine, both *in vitro* and *in vivo*.

## MATERIALS AND METHODS

*Chemicals.* Pyridoxal-5'-phosphate, 4-aminobutyrate, L-ornithine, L-glutamate, 2-oxoglutarate were purchased from Sigma Chemical Co., St. Louis, Mo., USA. N-meta-carboxyphenylpyridoxamine phosphate was synthesized as described (8).

Synthesis of 3-amino-1,5-cyclohexadienyl carboxylic acid: Birch reduction of benzoic acid yields 2,5-cyclohexadienoic acid (9). The ter-butyl ester was synthesized by treatment of the crude product of the Birch reduction with isobutylene in the presence of traces of $H_2SO_4$. The ester was purified by chromatography on alumina. A 10% excess of silver isocyanate in dichloromethane was added to the ester followed by the same amount of iodine. After stirring for one hour at 0°, then 3 hours at 25°, the isocyanate is trapped by addition of one equivalent para-methoxybenzyl alcohol. The carbamate formation is allowed to proceed overnight at room temperature. Dehydroiodination is achieved by treatment with one equivalent diazobicycloundecane in acetone. Ter-butyl, 3-(ter-butoxycarbonylamino)-1,5-cyclohexadiene carboxylate is then purified by silica gel chromatography. Hydrolysis of the two protecting groups is carried out in anisol by treatment with an excess of freshly distilled trifluoroacetic acid. The trifluoroacetate (TFA) salt of isogabaculine is crystallized by trituration with ether of the dry residue. Recrystallization from isopropanol-ether gives an analytically pure sample. The overall yield is about 30%; analysis C:42.69, H:4.00, N:5.28.

*Model studies with pyridoxal-5'-phosphate.* These reactions were essentially carried out as described by Rando for gabaculine (8). Isogabaculine was added to a solution of pyridoxal-phosphate in 0.1 μM phosphate buffer, pH = 7.0, at the appropriate temperature. Aliquots were withdrawn and analyzed for remaining free pyridoxal (absorption at 390 nm).

The products of the reaction were identified by thin layer electrophoresis, and comparison of ultraviolet and fluorescence spectra with spectra of authentic N-meta-carboxyphenyl-pyridoxamine phosphate.

*Enzymes: 4-aminobutyric acid aminotransferase.* GABA-T was purified from pig brain following the method described by John and Fowler (10). We are indebted to Dr. B. Lippert for the gift of a preparation having a specific activity of 4,6 μmole/min/mg protein. Enzyme activity was measured spectrophotometrically in a coupled assay with succinic semialdehyde dehydrogenase prepared from guinea pig kidney (B. Lippert *et al.*, to be published).

*Ornithine aminotransferase.* Orn-T (E.C. 2.6.1.13) was isolated and assayed as described previously (11).

*Glutamate decarboxylase.* We are indebted to Dr. M. Bouclier for the gift of a highly purified preparation of GAD (E.C. 4.1.1.15) from rat brain (method to be published). The enzyme was assayed radiometrically by the release of $^{14}CO_2$ from [1-$^{14}$C]glutamate (12).

*Animals.* Swiss albino mice (Charles River, France), weighing 20–25 g were used. Isogabaculine was given as a fresh aqueous solution (1 ml/100 g) by intraperitoneal injection. The animals were killed by decapitation at appropriate times. Brains were divided into halves by sagittal section; GABA concentration was measured in the acid extract of one half, and GAD and GABA-T were measured in the other half, as described previously (13).

## RESULTS

### *Reaction of Isogabaculine with Pyridoxal-5'-phosphate*

*Spectral changes.* When isogabaculine was allowed to react with an equimolar amount of pyridoxal-phosphate, the absorption peak of pyridoxal at 390 nm slowly disappeared, while a new absorption band at 315 nm appeared (Fig. 1). The presence of an isobestic point at 345 nm indicated the direct interconversion of one entity into another.

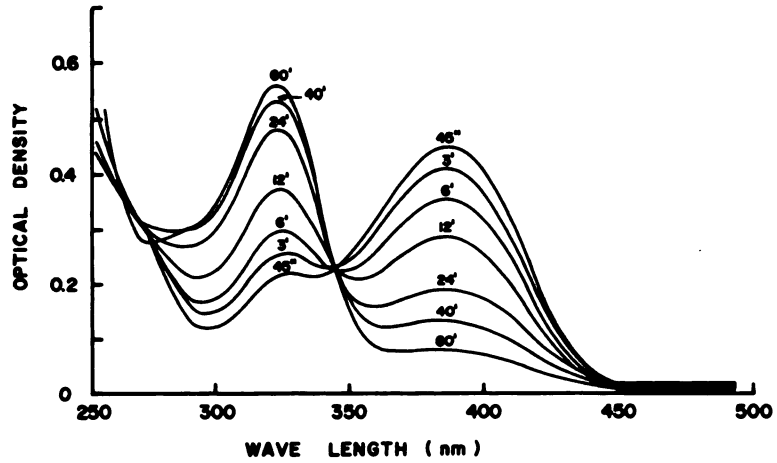


FIG. 1. *Spectral changes during the reaction of isogabaculine with pyridoxal-5'-phosphate*
Isogabaculine and pyridoxal phosphate (10 mM each) were allowed to react at 60° under nitrogen in the absence of light in 0.1 M phosphate buffer. At given times aliquots were withdrawn and diluted a hundred fold in the same buffer and the spectrum was recorded on an Acta III Beckman spectrophotometer.

*Characterization of the reaction product.* After the absorption band at 390 nm had nearly completely disappeared, the reaction mixture was analyzed by thin layer chromatography (silica gel G; BuOH:AcoH: $H_2O$, 60:20:20). Isogabaculine and pyridoxal-phosphate were present only in trace amounts and a new fluorescent product had appeared. There was no sign of meta-anthranilic acid formation. The new product had maximal ultraviolet absorption at 315 nm and the fluorescent spectrum was centered around 415 nm as for N-meta-carboxyphenyl-pyridoxamine phosphate (8). Thin layer electrophoresis (silica gel G; pH 5; pyridine acetate buffer) revealed that the new product co-migrated with authentic N-meta-carboxyphenyl-pyridoxamine phosphate.

*Activation energy.* When the temperature was increased, the rate of disappearance of pyridoxal-phosphate increased (Fig. 2A). An Arrhenius plot (Fig. 2B) showed that the activation energy of the reaction was 17.2 kcal, very similar to that of gabaculine (8).

## In vitro *Inhibition of Pyridoxal Enzymes*

*4-aminobutyric acid aminotransferase.* When purified GABA-T was incubated with isogabaculine, there was a time-dependent loss of enzyme activity that could not be reversed by extensive dilution or dialysis. The loss of enzyme activity followed pseudo-first-order kinetics almost until all the activity had disappeared (Fig. 3A). The kinetic data (Fig. 3B) showed that the process was saturable: $K_I$ = 20 μM and minimum half life was 0.8 min, corresponding to a kcat of 1.4 $10^{-2}$/sec. GABA and glutamate at 1 mM concentration slowed down the rate of inhibition, while pyridoxal phosphate was without effect.

*Other pyridoxal phosphate-dependent enzymes.* Orn-T was inhibited by isogabaculine in a time-dependent manner at the same concentrations of inhibitor as GABA-T (Fig. 4). GAD however was not affected, even at a concentration of 1 mM.

## *Effect of Isogabaculine on GABA Metabolism*

After a single injection of 0.36 mmole/kg of isogabaculine there was a rapid decrease of brain GABA-T activity. By four hours 85% of GABA-T was inhibited, and this level of inhibition was maintained at least for four days (Fig. 5A). GAD activity decreased very slowly, and presumably the decrease was not due to a direct interaction of isogabaculine with this enzyme. The concentration of GABA increased steadily for the first 20 hours after injection and reached approximately 12 times control val-

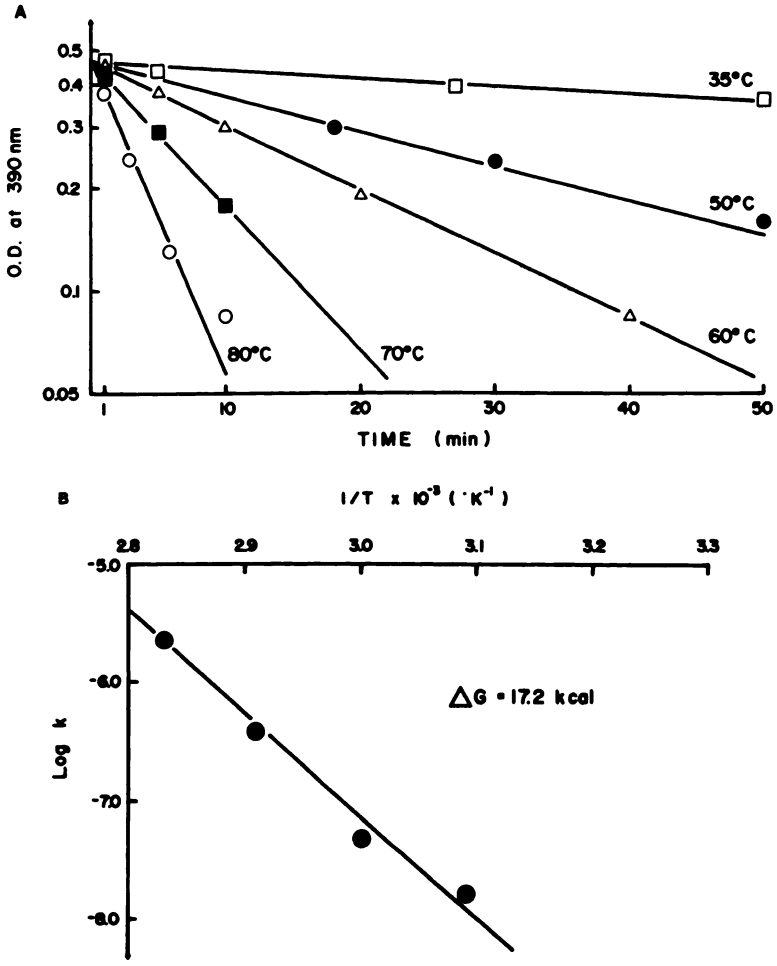


FIG. 2. *Temperature-dependence of the rate of reaction (A) and activation energy of the reaction (B) between isogabaculine and pyridoxal-5'-phosphate*

A. Isogabaculine (10 mM) was allowed to react with pyridoxal-5'-phosphate (0.1 mM) as in Figure 1 at different temperatures. At given intervals, one ml aliquots were withdrawn and the absorption at 390 nm was recorded. B. Data from Figure 2A are presented in an Arrhenius plot.

ues. The concentration of cerebral GABA started to decrease after 48 hours, but even after four days it was still seven times higher than control.

This dose of isogabaculine appeared to be toxic; all remaining animals died after the fourth day. Body temperature was extremely low (24°) and the animals were nearly comatose after day 3. After a dose of 0.21 mmole/kg (30 mg/kg free base), similar changes of GABA metabolism were found, but recovery of GABA-T and GAD activity started after day 2, and GABA levels were back to normal values by day 3 (data not shown).

The effects of isogabaculine on GABA metabolism were dose-dependent (Fig. 5B). A dose of 0.02 mmole/kg (5 mg/kg of TFA salt) has no effect on GABA-T or GAD activity and GABA concentration 8 hours after administration. Increasing the dose to 0.1 mmole/kg causes a highly significant reduction of GABA-T activity (−70%), a statistically nonsignificant reduction of GAD activity, and a fivefold elevation of brain GABA levels. Doses of 0.40 mmole/

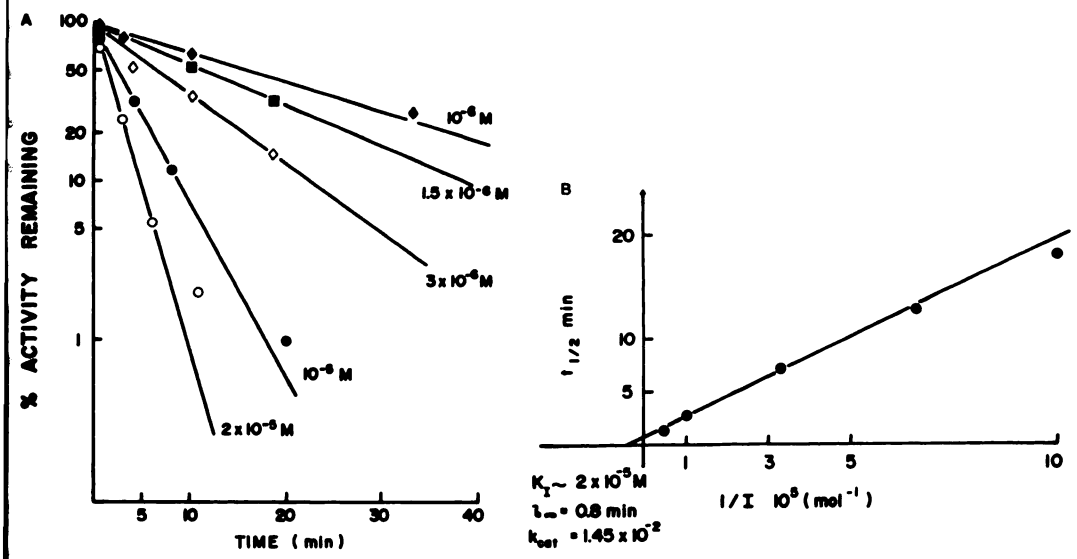


FIG. 3. *Time-dependent inactivation (A) and kinetic constants (B) of pig brain GABA-T by isogabaculine*

A. Purified GABA-T was incubated at 37° with different concentrations of isogabaculine in pyrophosphate buffer 0.1 M, pH 8.6, containing 10 mM mercaptoethanol. At different times after addition of isogabaculine, aliquots were withdrawn and assayed for remaining enzyme activity (4). B. The half-life of the enzyme activity at a given concentration of inhibitor determined from Figure 3A was plotted as a function of the reciprocal of this concentration (14).

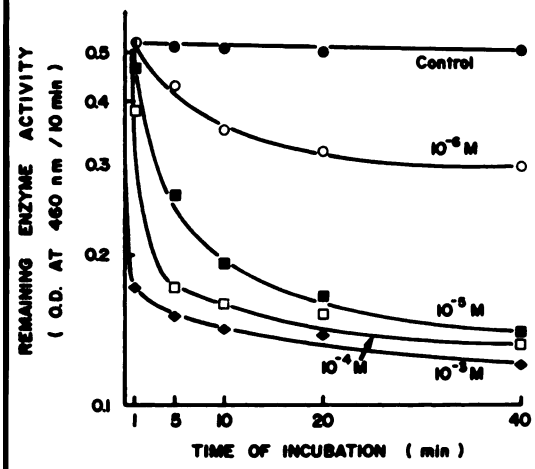


FIG. 4. *Time-dependent inhibition of Orn-T by isogabaculine*

Purified Orn-T was incubated with different concentrations of isogabaculine at 37° in potassium phosphate buffer 0.2 M, pH 8.0, as previously described (11). Aliquots were withdrawn and assayed at given time intervals for remaining enzyme activity.

kg, when GABA-T activity is almost completely inhibited, elevate GABA concentrations about 12-fold. The decrease of GAD activity is minimal and is never statistically significant.

## DISCUSSION

Isogabaculine reacts with pyridoxal phosphate to form N-meta-carboxyphenyl-pyridoxamine phosphate. The energy of activation is similar to that of gabaculine as calculated from the experimental values of Rando and Bangerter (8). It is a potent irreversible inhibitor of GABA-T *in vitro*. The affinity for GABA-T is 10 to 20 times lower than that reported for gabaculine and the half-life of enzyme activity at high concentrations of isogabaculine is much shorter so that the two compounds are almost equipotent in the μM range. Their enzyme selectivity is also very similar. Neither bacterial nor mammalian GAD is noticeably inhibited even at 100–1000 fold higher concentrations than those needed for GABA-T inhibition. However, both isogabaculine and gabaculine (11) irreversibly inhibit Orn-T. The two compounds also have very similar effects on GABA metabolism *in vivo*.

Gabaculine is a natural product extracted

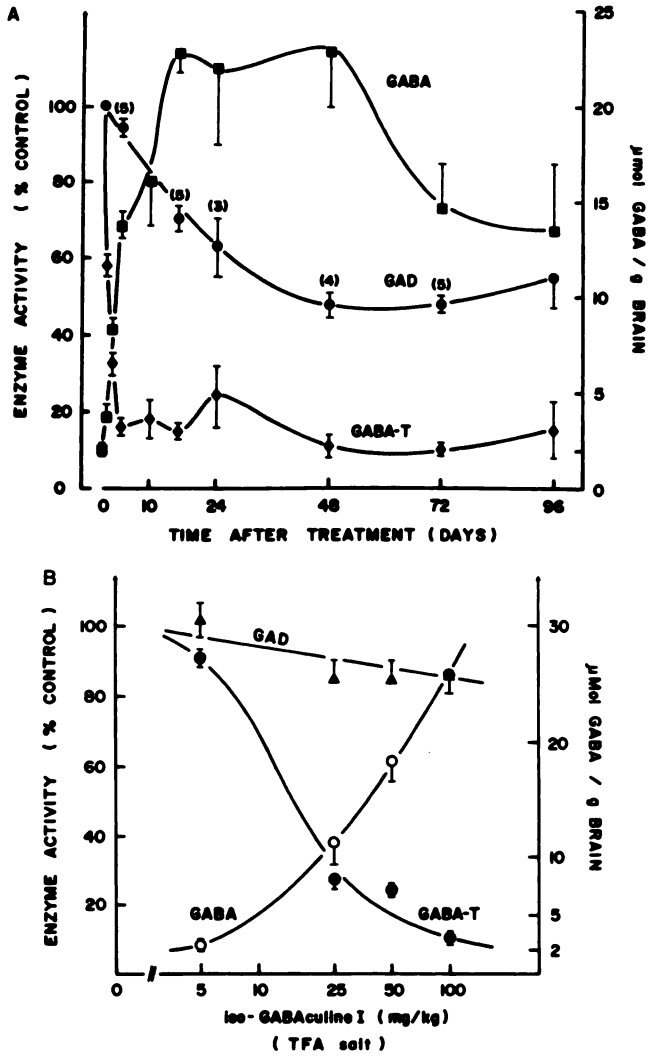


FIG. 5. *Time-dependent effect (A) and dose-dependent effects (B) of isogabaculine on cerebral GABA metabolism in mice*

A. Isogabaculine-trifluoroacetate salt was given i.p. to groups of 5 mice at a dose of 0.36 mmole/kg. At given time intervals the animals were decapitated and their brains processed as described previously (13) for the determination of brain GABA concentration and brain GABA-T and GAD activities. All values represent the mean ± SEM of 5 animals unless otherwise indicated. B. Groups of five mice were injected i.p. with four different doses of the isogabaculine salt. The animals were killed 8 hours later and brain GABA concentration, and GABA-T and GAD activities measured (13). All values are the mean ± SEM of 5 animals.

from Streptomyces toyocaensis (14); the synthetic isogabaculine has not yet been found in nature. Both compounds are reasonably stable under physiological conditions. In direct contrast the third double-bond isomer, 3-amino-1,4-cyclohexadienyl carboxylate, will probably be unstable and of no practical importance since its protected form, ter-butyl 3-(ter-butoxycarbonylamino)-1,4-cyclohexadiene carboxylate, is oxidized very rapidly in air to the meta-anthranilic acid derivative (15).

In pharmacological studies both gabaculine and isogabaculine are moderately effective anticonvulsive agents (16). The discovery of isogabaculine adds a new compound

to the steadily increasing list of products capable of interfering with GABA metabolism.

*Note added in proof*: The synthesis of 3-amino-1,4-cyclohexadienyl carboxylate, the third double-bond isomer of gabaculine has been described recently (Danis Hefsky, C., and F. M. Hershenson, *J. Org. Chem.* **44**: 1180–1181, 1979). Contrary to our expectations, the compound is reasonably stable in air. Biological properties have not yet been reported.

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