results are also consistent with the observed critical length for acquisition of secondary structure in angiotensin-II,[20] an octapeptide hormone, and agree with critical length estimates for helix formation in γ-ethyl L-glutamate oligopeptides.[21] Further studies are underway to elucidate the origins of the observed shifts, and to obtain quantitative estimates of the magnitudes of the shifts due to local electric fields.

References and Notes

(1) W. W. Bromer, L. G. Sinn, and O. K Behrens, *J. Am. Chem. Soc.*, **79**, 2807 (1955).
(2) M. H. Makman and E. W. Sutherland, *Endocrinology*, **75**, 127 (1964).
(3) F. Murad and M. Vaughn, *Biochem. Pharmacol.*, **18**, 1053 (1964).
(4) L. Birnbaumer and M. Rodbell, *J. Biol. Chem.*, **244**, 3477 (1969).
(5) K. Sasaki, S. Dockerill, D. A. Adamiak, I. J. Tickle, and T. Blundell, *Nature (London)*, **257**, 751 (1975).
(6) P. A. Srere and G. C. Brooks, *Arch. Biochem. Biophys.*, **129**, 708 (1969).
(7) B. Panijpan and W. B. Gratzer, *Eur. J. Biochem.*, **45**, 547 (1974).
(8) P. Y. Chou and G. D. Fasman, *Biochemistry*, **14**, 2536 (1975).
(9) J. B. A. Ross, Dissertation, University of Washington, Seattle, 1976.
(10) M. Sharnoff, *J. Chem. Phys.*, **46**, 3263 (1967).
(11) A. L. Kwiram, *Chem. Phys. Lett.*, **1**, 272 (1967).
(12) A. L. Kwiram, *MTP Int. Rev. Sci., Phys. Chem.: Ser. One*, **4**, 271 (1972).
(13) The zfs arise from the anisotropic interaction of the two unpaired electrons, and this depends intimately upon the properties of the molecular orbitals involved in the triplet state.
(14) This assumes D,E > 0.
(15) J. Zuclich, J. U. Von Schütz, and A. H. Maki, *Mol. Phys.*, **28**, 33 (1974).
(16) K. W. Rousslang and A. L. Kwiram, *Chem. Phys. Lett.*, **39**, 226 (1976).
(17) K. W. Rousslang and A. L. Kwiram, *Chem. Phys. Lett.*, **39**, 231 (1976).
(18) L. Peller, *J. Phys. Chem.*, **63**, 1194 (1959).
(19) B. H. Zimm and J. K. Bragg, *J. Chem. Phys.*, **31**, 526 (1959).
(20) S. Fermandjian, J. L. Morgat, and P. Fromageot, *Eur. J. Biochem.*, **24**, 252 (1971).
(21) M. Goodman, A. S. Verdini, C. Toniolo, N. D. Phillips, and F. A. Bovey, *Proc. Natl. Acad. Sci. U.S.A.*, **64**, 444 (1969).

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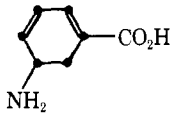
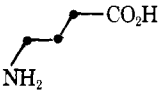
The Irreversible Inhibition of Mouse Brain γ-Aminobutyric Acid (GABA)-α-Ketoglutaric Acid Transaminase by Gabaculine

Sir:

There are a large number of natural products that are specific, irreversible enzyme inhibitors. These molecules are of diverse structural types but are linked by a common mechanism of action.[1] This general mechanism of action requires these molecules, which are chemically unreactive, to be substrates for the target enzyme. In the process of catalytic turnover, the enzyme becomes inactive.[2] That is, the target enzyme catalyzes its own destruction. The fact that these molecules are chemically unreactive before turnover is the key to the specificity of these inhibitors. We would like to report here a further example of a naturally occurring irreversible inhibitor of this type. Specifically, we show that gabaculine (5-amino-1,3-cyclohexadienylcarboxylic acid), a natural product isolated from *Streptomyces toyocaensis*,[3] is a potent irreversible inhibitor of mouse brain, pyridoxal phosphate linked γ-aminobutyric acid (GABA)-α-ketoglutarate transaminase and that the mechanism of action of this inhibitor requires its catalytic turnover.



gabaculine



GABA

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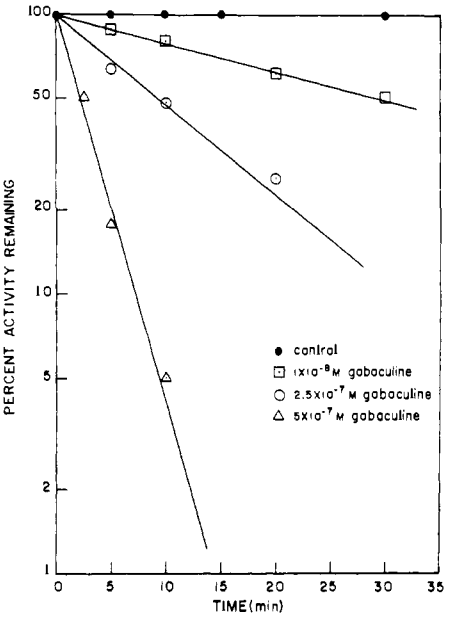


Figure 1. Irreversible inhibition of GABA-transaminase by gabaculine; 0.05 units of mouse brain enzyme (specific activity = 0.03 units/mg) was incubated with gabaculine at the indicated concentrations (based on *l*-gabaculine) in 0.1 ml of 0.1 M potassium phosphate buffer, pH 8.3 at 37 °C. A unit of enzyme is defined as the amount of enzyme required to catalyze the formation of 1 μmol of product per min under standard conditions.[5] At the indicated times aliquots of the enzyme were removed and the remaining activity determined by the method of Wu which uses a coupled assay with glutamate dehydrogenase and acetyl-NAD.[5] The activity of the inactive enzyme would not be increased in the slightest by extended dialysis against the phosphate buffer.

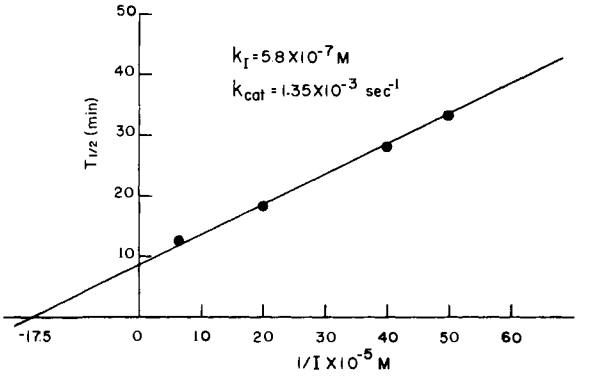


Figure 2. Determination of K_I and k_{cat} for gabaculine. The rates of transaminase inhibition were determined for the indicated concentrations of gabaculine at 15 °C. Assuming the scheme

$$E + I \overset{K_I}{\rightleftarrows} EI \xrightarrow{k_{cat}} EI' \xrightarrow{k_{inh}} E - I'$$

with $d(EI)/dt = 0$, $I \gg E$ and $k_{inh} \gg k_{cat}$ the following equation is derived $t_{1/2} = (0.69/k_{cat} + 0.69/k_{cat})K_I/I$[4]. A plot of $t_{1/2}$ vs. $1/I$ yields a $K_I = 5.8 \times 10^{-7}$ M and a $k_{cat} = 1.35 \times 10^{-3}$ s^{-1} at 15 °C.

In the presence of gabaculine the mouse brain enzyme is progressively and irreversibly inactivated (Figure 1). The inhibited enzyme cannot be reactivated by gel filtration or by extended dialysis against buffer. Mercaptoethanol (10 mM) has no effect on the rate of inactivation suggesting that an affinity labeling mode of inactivation is not occurring. Furthermore, derivatives of gabaculine incapable of being substrates for the enzyme are not inhibitors of it. For example, the *tert*-butylcarbamate derivative of gabaculine in the micromolar range is neither an irreversible nor a competitive inhibitor of the enzyme. In addition, synthetic *d,l*-gabaculine has one-half the activity of the naturally occurring *l*-isomer.[3] These results, taken together, argue against a direct chemical

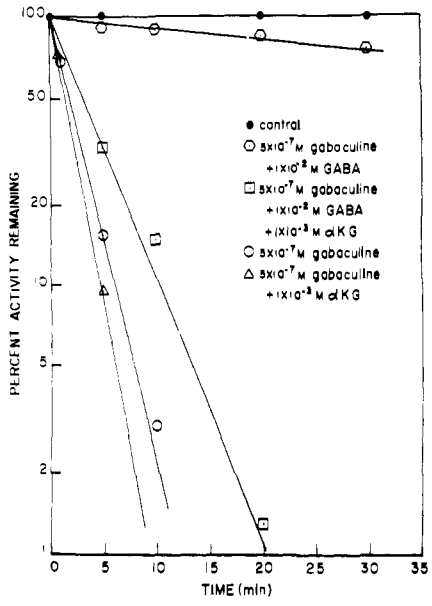


Figure 3. Effect of GABA and α-ketoglutarate on the rate of inactivation; 0.05 units of enzyme was incubated with 5×10^{-7} M gabaculine alone and with the same concentration of gabaculine plus 10^{-2} M GABA, plus 10^{-2} M GABA and 10^{-3} M α-ketoglutarate, and plus 10^{-3} M α-ketoglutarate. In addition there was a control with no addition. At the indicated times aliquots were removed and the remaining enzymatic activity was determined.

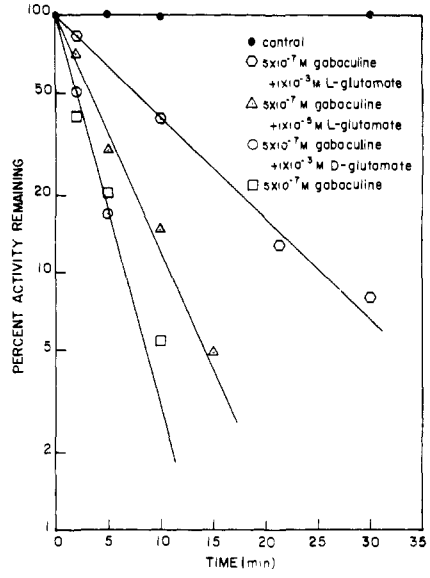


Figure 4. Effect of D- and L-glutamic acid on the rate of inactivation; 0.05 units of enzyme was incubated with 5×10^{-7} M gabaculine alone, with 10^{-3} M and 10^{-5} M L-glutamate, and with 10^{-3} M D-glutamate. There was also a control with no additions. At the indicated times aliquots were removed, and the remaining enzymatic activity was determined.

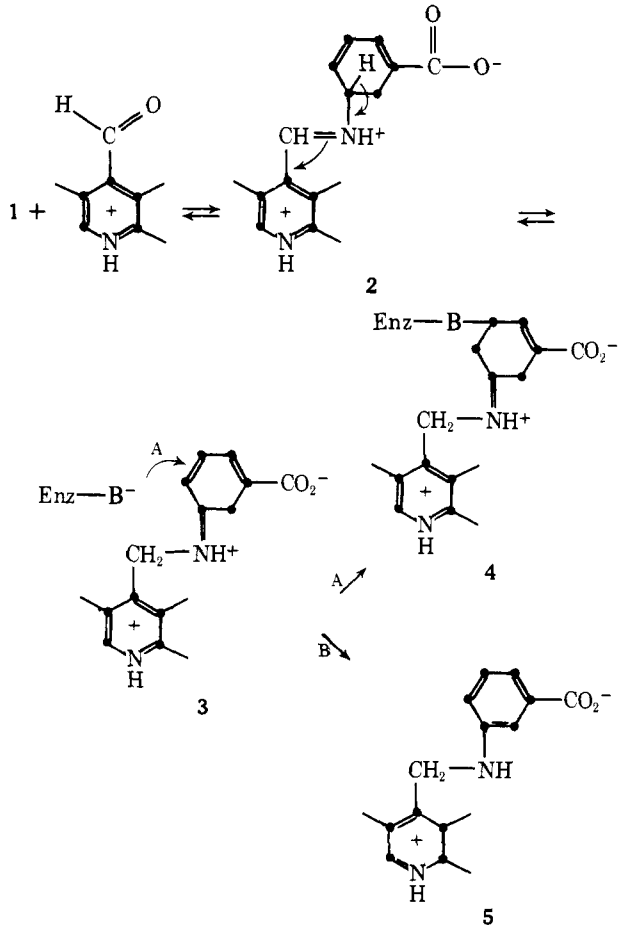
reaction occurring between gabaculine and the enzyme, but rather suggest that catalytic conversion of the inhibitor occurs.

The K_I for gabaculine and the k_{cat} for its turnover can be obtained by plotting $t_{1/2}$ vs. $1/I$ at various inhibitor concentrations.[4] This plot is shown in Figure 2. The measured K_I is 5.8×10^{-7} M and the k_{cat} is 1.35×10^{-3} s^{-1} at 15 °C. It is noteworthy that the K_I for gabaculine is some 10^3 lower than the K_M for the normal substrate GABA. The rigid conformation assumed by the GABA moiety of gabaculine must mimic the conformation of GABA at the active site. No such potent inhibition was observed with *m*-anthranilic acid so that the interaction between gabaculine and the enzyme must be quite specffic.

Substrates for the enzyme have a marked effect on the rate of inactivation. GABA itself strongly retards the rate when the enzyme is preincubated with it (Figure 3). α-Ketoglutarate, on the other hand, increases the rate of inactivation compared to just enzyme and gabaculine (Figure 3). These experiments suggest that gabaculine is an inhibitor of the pyridoxal form of the enzyme and not of the pyridoxamine form. That this is indeed the case can be further shown in Figure 3 where the protective effect of GABA is abolished when α-ketoglutarate is added. This latter reagent, of course, converts the pyridoxamine form of the enzyme (GABA protected) into the pyridoxal form. Nonsubstrate α-ketoacids, such as α-ketobutyrate, have no effect on the rate of inactivation. The protective effect of substrate amines can also be demonstrated with D- and L-glutamate. As shown in Figure 4, only the substrate L-glutamate protects the enzyme against gabaculine induced irreversible inhibition. α-Ketoglutarate abolishes this protective effect (not shown). The fact that the enzyme must be in the pyridoxal form prior to its being inactivated is consistent with the notion that gabaculine is a substrate for the enzyme which inactivates the enzyme as a consequence of or during catalytic conversion.

If catalytic turnover of gabaculine is rate-limiting in the inactivation process, then the pH vs. rate of inhibition profile should be similar to that for normal substrate turnover. This is the case. The pH vs. rate of inactivation profile is shown in Figure 5. The maximum is shifted about 0.5 pH units down from the pH rate maximum for GABA turnover but is otherwise identical with it.[5] Furthermore, there is a deuterium isotope effect (K_H/K_D) of 2.1 on the rate of inhibition of the enzyme when inhibited by 4,5-dideuterogabaculine.[6] This demonstrates that the rate-limiting step in the inactivation is the cleavage of the α-C-H bond.

Two straightforward mechanisms can be considered for the inactivation process and these are shown below:



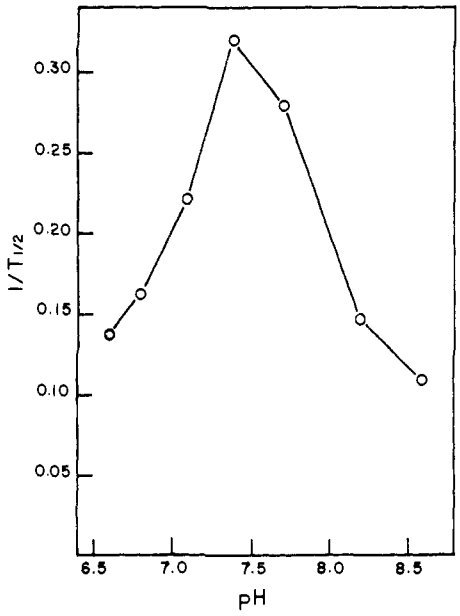


Figure 5. pH vs. rate of inactivation; 0.05 units of enzyme was incubated with 5×10^{-7} *M* gabaculine in 0.1 *M* potassium phosphate buffer at pH's 6.6, 6.8, 7.1, 7.4, and 7.8 and potassium pyrophosphate buffer at pH's 8.2 and 8.6 at 37 °C. The rates of inactivation of the enzyme were measured and the $t_{1/2}$'s determined. $1/t_{1/2}$ is plotted vs. pH above. The same curve is generated at saturating levels of gabaculine.

The initial step in the inactivation process leads to the rate limiting formation of the enzyme bound, transaminated product **3.** Sequence A would involve a Michael addition of an active-site nucleophile to this activated intermediate to generate the irreversibility inhibited enzyme **4.** This mode of inhibition has precedent in the irreversible inactivation of pyridoxal linked aspartate aminotransferase by β,γ-unsaturated amino acids.[7] Sequence B would involve the simple aromatization of **3** to yield the *m*-anthranilic acid derivative **5.** This process would be expected to occur spontaneously if a Lewis base happens to be adjacent to the 2-C-H bond of **3** in the enzyme-inhibitor complex. This process would also yield an irreversibly inactivated enzyme because this complex would remain enzyme bound. Pyridoxal phosphate itself strongly resists resolution from the native holoenzyme. Further experiments are in progress to decide which of these two mechanisms, if either, best accomodate the data.

Both mechanisms require the direct irreversible inhibition of the enzyme prior to the dissociation of the enzyme inhibitor complex **3.** We can rule out other mechanisms that would produce a diffusible inhibitor in two ways. First of all, exogenous trapping agents such as mercaptoethanol have no effect on the rate of inactivation. Secondly, successive additions of enzyme to an excess of inhibitor all show the same rates of inhibition.[6] A mechanism that postulates a reactive intermediate in solution would predict that each succeeding addition of enzyme would be inhibited more rapidly than the previous one.

Gabaculine is likely to be useful in defining the physiological roles of the inhibitory neurotransmitter GABA in vivo. We have found that gabaculine irreversibly inhibits mouse brain enzyme in vivo. It should be noted that the most specific irreversible inactivator of GABA transaminase to date, 4-amino-5-hexynoic acid, a very cleverly designed inhibitor which also functions by a mechanism requiring catalytic turnover, is two to three orders of magnitude less potent than is gabaculine in vitro.[4]

In conclusion, we have demonstrated that the natural product gabaculine is a potent, specific irreversible inhibitor of mammalian GABA transaminase both in vitro and in vivo. This compound is a further example of a naturally occurring irreversible inhibitor that functions by a novel mechanism requiring the enzyme to catalyze its own destruction.

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References and Notes

(1) R. R. Rando, *Acc. Chem. Res.*, **8**, 281 (1975).
(2) R. R. Rando, *Science*, **185**, 320 (1974).
(3) H. Mishima, H. Kurihara, K. Kobayashi, S. Miyazawa, and A. Terahara, *Tetrahedron Lett.*, 7, 537 (1976).
(4) M. J. Jung and B. W. Metcalf, *Biochem. Biophys. Res. Commun.*, **67**, 301 (1975); R. Kitz and I. B. Wilson, *J. Biol. Chem.*, **237**, 3245 (1964).
(5) J. Y. Wu in "GABA in Nervous System Function", E. Roberts, T. N. Chase, and D. B. Tower, Ed., Raven Press, New York, N.Y., 1976.
(6) R. R. Rando, unpublished experiments.
(7) R. R. Rando, *Biochemistry*, **13**, 3859 (1974); R. R. Rando, *Nature (London)*, **250**, 586 (1974); R. R. Rando, N. Relyea, and L. Cheng, *J. Biol. Chem.*, in press.

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Additions and Corrections

Stereochemistry of Metal Anion Substitutions on Carbon [*J. Am. Chem. Soc.*, **94**, 7205 (1972)]. By G. S. KOERMER, M. L. HALL, and T. G. TRAYLOR,* Department of Chemistry, Revelle College, University of California, San Diego, La Jolla, California 92093.

The last entry in Table I is incorrect. No 4-camphyltrimethyltin was obtained. We have since found that, whereas bridgehead bromides react readily with Me_3SnLi, bridgehead chlorides are unreactive.

Photochemical Rearrangement of β-*tert*-Butylstyrenes. Stereochemistry [*J. Am. Chem. Soc.*, **95**, 3031 (1973)]. By STEPHEN S. HIXSON* and TIMOTHY P. CUTLER, Department of Chemistry, University of Massachusetts, Amherst, Massachusetts 01002.

Substituent Effects on π-π^* Rearrangements. Methyl Migration in β-*tert*-Butylstyrenes [*J. Am. Chem. Soc.*, **95**, 3032 (1973)]. By STEPHEN S. HIXSON* and TIMOTHY P. CUTLER, Department of Chemistry, University of Massachusetts, Amherst, Massachusetts 01002.