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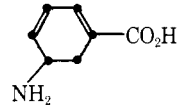
Mechanism of the Irreversible Inhibition of γ-Aminobutyric Acid–α-Ketoglutaric Acid Transaminase by the Neurotoxin Gabaculine†

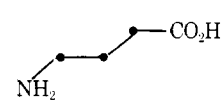
R. R. Rando

ABSTRACT: Gabaculine (5-amino-1,3-cyclohexadienylcarboxylic acid), a naturally occurring amino acid isolated from *Streptomyces toyocaenis,* is an irreversible inhibitor of bacterial pyridoxal phosphate linked γ-aminobutyric acid–α-ketoglutaric acid transaminase with a $t_{1/2}$ (25 °C) of 9 min at 3×10^{-7} M. Gabaculine is a substrate for γ-aminobutyric acid transaminase. The measured K_I is 2.86×10^{-6} M, and the k_{cat} for its turnover is 1.15×10^{-2} s^{-1} at 25 °C. When gabaculine is transaminated by the enzyme, it is converted to a cyclohexatrienyl system with one exo double bond. Upon spontaneous aromatization, this high energy intermediate is transformed into a stable *m*-anthranilic acid derivative (*m*-carboxyphenylpyridoxamine phosphate), which results in the covalent and irreversible modification of the cofactor. This adduct is bound tightly to the active site of the enzyme and can be liberated under denaturing conditions.

Naturally occurring irreversible enzyme inhibitors often function by a mechanism that requires their catalytic turnover by the target enzyme prior to the inhibition step (Rando, 1975). If a chemically reactive intermediate is generated, the inactivation of the enzyme can result from a reaction of this intermediate with an active-site residue or cofactor. Thus, in the process of catalytic turnover the enzyme becomes inactivated. The fact that these molecules are chemically unreactive before turnover is the key to their specificity. 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), **1,** a natural



gabaculine



γ-aminobutyric acid

product isolated from *Streptomyces toyocaenis* (Mishima et al., 1976), is a potent irreversible inhibitor of bacterial, pyridoxal phosphate linked, γ-aminobutyric acid–α-ketoglutaric acid transaminase, and that the mechanism of action of this inhibitor involves its enzymatic transamination to form **2** followed by a subsequent aromatization step to generate *m*-carboxyphenylpyridoxamine phosphate (CPPp) (**3**). The net effect of this process is to covalently link the inhibitor to the cofactor. Although the mechanism of inactivation of γ-amino-

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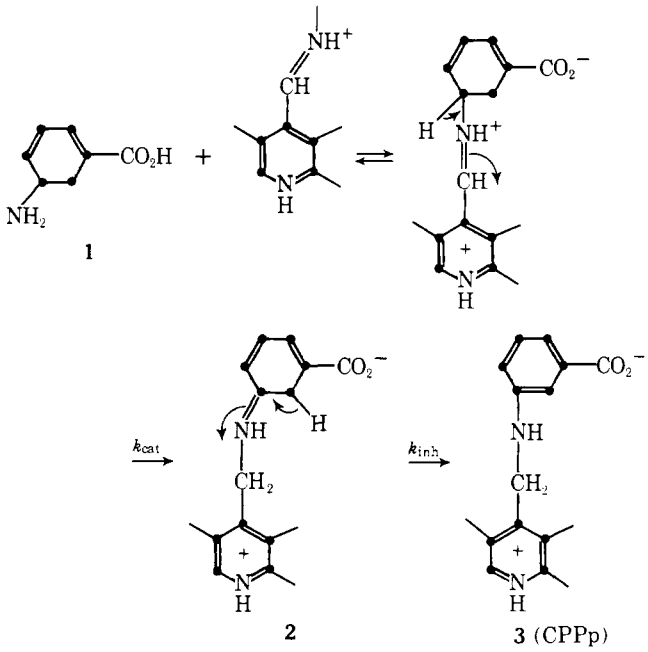
butyric acid transaminase by gabaculine is a member of the class of irreversible enzyme inhibitors described above, the aromatization step makes it quite unlike any previously identified member of this class.



1 2 3 (CPPp)

Experimental Procedure

Materials and Methods

Partially purified γ-aminobutyric acid transaminase (2.3 units/mg) from *Pseudomonas fluorescens* and commercial enzyme (0.56 unit/mg) from Sigma Chemical Co. were used (Scott and Jakoby, 1959; Jakoby and Scott, 1959). A unit of activity is defined as the amount of transaminase allowing the formation of 1 μmol of reduced pyridine nucleotide per min in the standard assay system (Scott and Jakoby, 1959; Jakoby and Scott, 1959). The standard assay system involves incubating the transaminase with γ-aminobutyric acid, α-ketoglutarate, NADP, and excess succinic semialdehyde dehydrogenase (Scott and Jakoby, 1959; Jakoby and Scott, 1959). The resulting increase in absorbance at 340 nm is directly related to the activity of the transaminase. We have also measured the transaminase activity by coupling it to glutamate dehydrogenase according to the method of Wu (1976). Both assay systems gave equivalent results.

The kinetic scheme used to analyze the inactivation data was derived from the work of Kitz and Wilson (1964) as modified by Jung and Metcalf (1975). The final equation used is

$$t_{1/2} = \frac{0.69}{k_{cat}} + \frac{0.69}{k_{cat}} K_{I/I}$$

and is easily derived from the following equation

$$E + I \overset{K_I}{\rightleftharpoons} EI \overset{k_{cat}}{\longrightarrow} E \cdot I' \overset{k_{inh}}{\longrightarrow} E\text{-}I$$

where it is assumed that (EI) is constant, (I) ≫ (E), and $k_{inh} \gg k_{cat}$. By plotting $1/I$ vs. $t_{1/2}$ one obtains the first-order rate constant k_{cat} and the dissociation constant for the EI complex.

Determination of K_I and k_{cat} for Gabaculine Induced Irreversible Inhibition of γ-Aminobutyric Acid Transaminase. Enzyme (0.4 unit) was incubated with gabaculine at 1×10^{-6}, 3×10^{-7}, and 1×10^{-7} M in 0.1 mL of pH 8.6 buffer at 25 °C. The rates of inhibition were measured in the usual way and the $t_{1/2}$ (halftimes) were determined at the different inhibitor concentrations. $t_{1/2}$ was plotted against $1/I$ to give a straight line. From the y intercept and the slope of the line, values of $k_{cat} = 1.15 \times 10^{-2}$ s^{-1} and $K_I = 2.86 \times 10^{-6}$ were obtained.

Effects of D- and L-Glutamic Acid on the Rate of Inactivation. These experiments were also run identically with those in Figure 2. Enzyme was preincubated with 10^{-3} M L- and D-glutamate. Gabaculine at 5×10^{-7} M was added and the rates of inactivation were measured. Complete protection against inactivation was observed when L-glutamate was added, whereas D-glutamate did not afford any protection. The protective effect of L-glutamate was abolished by the addition of 10^{-5} M α-ketoglutarate.

pH vs. Rate of Inactivation Studies. Sodium phosphate buffers (0.1 M) of pHs 6.5, 7.0, 7.5 and 0.1 M sodium pyrophosphate buffers of pHs 8.0, 8.3, 8.6, and 9.1 were made up. Enzyme (0.4 unit) was added to 0.5 mL of these buffers along with 3×10^{-7} M gabaculine. The remaining enzymatic activities were determined at times in the usual way using the coupled assay systems. The rates of inactivation were determined at the different pHs. The maximum rate of inactivation occurred at pH 8.1.

Syntheses

[2-^{3}H]Gabaculine. This synthesis is essentially identical with the one provided for the preparation of the unlabeled material (Mishima et al., 1976).

Freshly prepared silver cyanate (1.43 g, 9.2 mmol) was added to a solution of 1.0 g (7.1 mmol), [2-^{3}H]methyl 1,4-cyclohexadienylcarboxylate (specific activity 17.8 mCi/mmol) (KOR Isotopes) in 8 mL of methylene chloride. Solid iodine (1.8 g, 7.1 mmol) was added with stirring at −5 °C. After the addition was completed, the mixture was stirred at 0 °C for 1 h and at room temperature for 3 h. The mixture was filtered through Celite and the filtrate was evaporated under reduced pressure. *p*-Methoxybenzyl alcohol (1.0 g) and 50 mg of diisobutyltin dilaurate were added and the mixture was stirred overnight. The oily adduct weighed 4.4 g. This compound was dehydroiodinated by treating it with 1,4-diazabicyclo[2.2.2]-octane (0.7 g, 6 mmol) in 50 mL of acetone. The mixture was stirred at room temperature for 2 h and then diluted with ether. The ethereal solution was washed with water and concentrated in vacuo. The oily product weighed 3.5 g.

Trifluoroacetate Salt of [2-^{3}H]Methyl-5-amino-1,3-cyclohexadienylcarboxylate. To an ice-cooled solution of the previous oily mixture (3.5 g) in 3 mL of anisole, 3 mL of trifluoroacetic acid was added in a dropwise fashion. After the addition was complete, the reaction mixture was allowed to remain at 10 °C for 1 h. The reaction mixture was concentrated under reduced pressure (2 mmHg) to remove the excess anisole and trifluoroacetic acid. The oily residue was cooled in an ice bath and dry ether was dropped in with stirring. The product crystallized (0.9 g) and was filtered and recrystallized from ethyl acetate–ether. The melting point of the compound was 142–143 °C.

tert-Butylcarbamate of [2-^{3}H]Methyl-5-amino-1,3-cyclohexadienylcarboxylate. The trifluoroacetate salt (0.5 g) made in the previous step was dissolved in 2 mL of dimethylformamide. With stirring, 1 mL of triethylamine and 1 mL of *tert*-butoxycarbonyl azide were added. The solution was stirred overnight and then evaporated in vacuo affording 0.55 g of the *tert*-butylcarbamate as an oil.

Hydrolysis of the tert-Butylcarbamate of [2-^{3}H]Methyl-5-amino-1,3-cyclohexadienylcarboxylate. The *tert*-butyl-

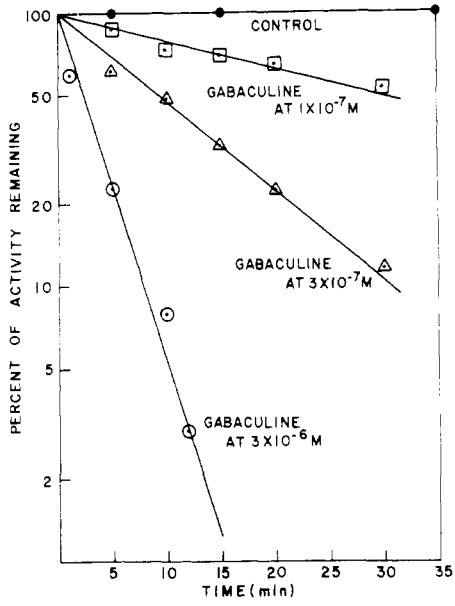


FIGURE 1: Irreversible inhibition of γ-aminobutyric acid transaminase by gabaculine at different concentrations. GABA-transaminase, 0.39 unit (0.56 unit/mg of protein), was incubated at 25 °C in 0.5 mL of a 0.1 M sodium pyrophosphate buffer, pH 8.6, with the D,L-gabaculine at the concentrations shown (concentrations calculated with the knowledge that only the 1-enantiomer of gabaculine is active). At various times, 10-μL aliquots of the solution were removed and diluted 100-fold in a cuvette with 1 mL of the pyrophosphate buffer, and the remaining activity of the enzyme was determined. ● refers to the activity of the untreated control, and ⊡ refers to the remaining activity of the enzyme treated with 1×10^{-7} M gabaculine, ▲ with 3×10^{-7} M gabaculine, and ⊙ with 3×10^{-6} M gabaculine. Gel filtration of the inactivated enzyme through Sephadex G-15 did not lead to any activity gain nor did extended dialysis against the phosphate buffer.

carbamate methyl ester (0.55 g) was treated with 0.2 g of sodium hydroxide in 2 mL of methanol and 1 mL of water. The solution was stirred at room temperature for 3 h, acidified to pH 3 with dilute hydrochloric acid, and evaporated to dryness in vacuo. The solid carbamate was dissolved in ether and then recrystallized from the same solvent. The product melted at 147–148 °C and weighed 0.33 g.

Hydrolysis of the tert-Butylcarbamate by [2-^{3}H]-5-Amino-1,3-cyclohexadienecarboxylic Acid. The acid (0.33 g) was dissolved in 7 mL of methanol and treated with 3.5 mL of 5% aqueous hydrochloric acid. The solution was stirred at room temperature for 18 h. Evaporation and the solution led to the recovery of 0.15 g of [2-^{3}H]gabaculine hydrochloride. Recrystallization of this compound from methanol/water led to the pure product melting at 197–199 °C. The free amino acid (*dl*-[2-^{3}H]gabaculine) was eluted from SP Sephadex C-25 with 0.2 N NH_4OH. The ultraviolet, NMR,[1] and infrared spectral characteristics of *dl*-[2-^{3}H]gabaculine and authentic gabaculine were identical. In addition, the tritiated material showed identical thin-layer chromatographic and paper electrophoretic behavior to the authentic material. The compound had a specific activity of 17.6 mCi/mmol.

4,5-Dideuteriogabaculine. This synthesis was carried out identically with the one reported for [2-^{3}H]gabaculine, except the starting material was methyl-4,5-dideuterio-1,4-cyclohexadienecarboxylate (Craig and Fowler, 1961). The final product (4,5-dideuteriogabaculine) and gabaculine had identical ultraviolet spectra. NMR analysis of the dideuterio compound synthesized in this way showed that the 5-position contained 80–90% deuterium depending on the preparation. The compound melted at 195–196 °C.

m-Carboxyphenylpyridoxamine Phosphate (CPPp) and m-Carboxyphenylpyridoxamine (CPP). Both compounds were synthesized by the published procedure which involved reducing the Schiff base formed between *m*-anthranilic acid and either pyridoxal phosphate or pyridoxal with sodium borohydride (Rando and Bangerter, 1977a). The compounds were fluorescent and homogeneous by thin-layer chromatography and paper electrophoresis.

Labeling of γ-Aminobutyric Acid Transaminase with [2-^{3}H]Gabaculine. The enzyme was radioactively labelled by incubating it with [2-^{3}H]gabaculine. The excess inhibitor was removed in two ways which gave equivalent results. Dialysis against 10^3 volumes of distilled water with five changes completely removed all unreacted inhibitor. In addition, high pressure filtration of the inhibited enzyme through an Amicon Model 8MC ultrafiltration system with a PM-10 filter resulted in the retention of labeled enzyme. After four washes with distilled water, all excess inhibitor was removed. [^{3}H]CPPp was removed from the filter by treating it with 2 mL of a 0.1% solution of trypsin or by boiling the filter in 2 mL of distilled water.

Radioactivity determinations were conducted on a Beckman LS-330 liquid scintillation counter using Aquasol (New England Nuclear Inc.) as the scintillation cocktail.

Electrophoretic separations of CPPp and CPP were conducted in a Durrum cell (Beckman Instruments, Inc) using a formic/acetic acid buffer, pH 1.8 (25 mL of formic acid + 80 mL of acetic acid per L). CPP and CPPp were localized by their intense fluorescence. Thin-layer chromatographic separations were conducted on EM silica gel plates without indicator. The eluent was BuOH–HOAc–H_2O (2:2:1). The plates were scraped and incubated with 0.5 mL of 1 M HCl for 15 min before adding 10 mL of Aquasol and counting. The paper electrophoresis strips were dried out, cut into smaller 2-cm strips, and eluted with 0.5 mL of water before adding 10 mL of Aquasol and counting.

Enzyme kinetics were conducted on a Gilford Model 240 spectrophotometer. γ-Aminobutyric acid, α-ketoglutaric acid, α-ketobutyric acid, D-glutamic acid, L-glutamic acid, succinic semialdehyde dehydrogenase, pyridoxal phosphate, trypsin, and alkaline phosphatase were all products of the Sigma Chemical Co. *N*-Methyl-γ-aminobutyric acid was a product of ICN Pharmaceuticals Inc. [2-^{3}H]Methyl-1,4-cyclohexadienecarboxylate was a product of KOR Isotopes Inc., Cambridge, Mass.

Results

Irreversible Inactivation of γ-Aminobutyric Acid Transaminase by Gabaculine. Incubation of γ-aminobutyric acid transaminase with D,L-gabaculine at various concentrations led to the irreversible inhibition of the enzyme, Figure 1. The control did not lose activity for up to approximately 1 h under the experimental conditions used. At 3 μM inhibitor, the $t_{1/2}$ for the enzyme is 3.25 min at 25 °C. The kinetics of the inactivation process are pseudo-first-order in enzymes and the inhibited enzyme was not reactivated in the least by continued dialysis against the pyrophosphate buffer (pH 8.6) or by gel filtration through Sephadex G-15.

The possibility that gabaculine was inactivating the enzyme by a direct alkylation process was shown to be unlikely by demonstrating that (1) only one of the two enantiomers of gabaculine was active as an inhibitor; (2) the *tert*-butylcar-

[1] Abbreviations used: NMR, nuclear magnetic resonance; $NaDodSO_4$, sodium dodecyl sulfate; CPPp, *m*-carboxyphenylpyridoxamine phosphate; CPP, *m*-carboxyphenylpyridoxamine; GABA, γ-aminobutyric acid.

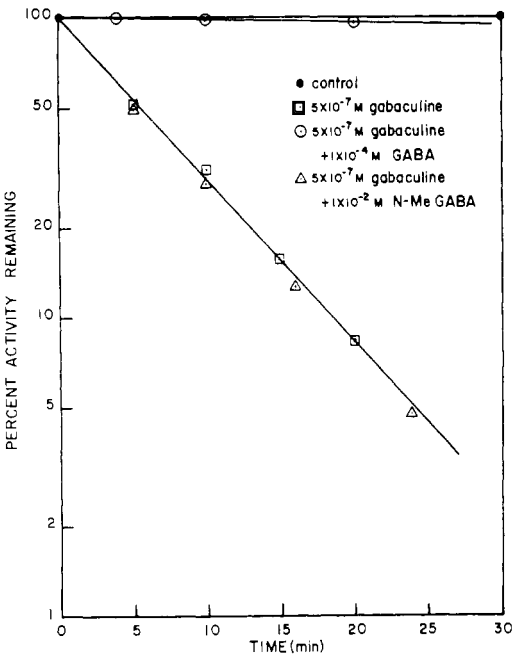


FIGURE 2: Effect of γ-aminobutyric acid and *N*-methyl-γ-aminobutyric acid on rate of inactivation. Into each of four test tubes was added 0.45 mL of 0.1 M sodium pyrophosphate at pH 8.6 and 0.4 unit of transaminase. The total volume was 0.5 mL. The first tube served as a control. To the second was added 5×10^{-7} M gabaculine, to the third 10^{-4} M γ-aminobutyric acid followed after 5 min with 5×10^{-7} M gabaculine, and to the fourth 10^{-2} M *N*-methyl-γ-aminobutyric acid followed after 5 min with 5×10^{-7} M gabaculine. Aliquots of the samples were assayed for enzymatic activities at the times indicated by the standard assay system described above. ● refers to the control, ⊙ to gabaculine plus γ-aminobutyric acid, ⊡ to gabaculine alone, and △ to gabaculine plus *N*-methyl-γ-aminobutyric acid.

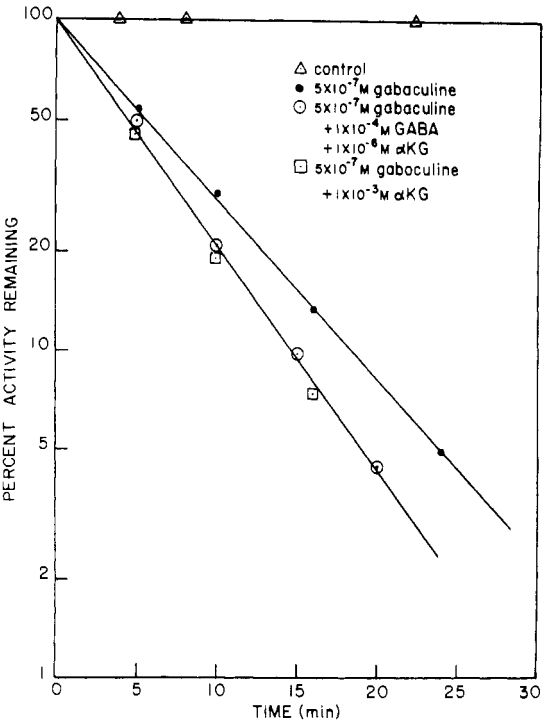


FIGURE 3: Abolition of γ-aminobutyric acid protection by α-ketoglutaric acid. These experiments were run identically with those shown in Figure 2. △ refers to the control, ● to gabaculine at 5×10^{-7} M, ⊙ to enzyme preincubated with 10^{-4} M γ-aminobutyric acid to which 5×10^{-7} M gabaculine and 10^{-6} α-ketoglutarate were added, and ⊡ to the enzyme incubated with 5×10^{-7} M gabaculine and 10^{-6} M α-ketoglutaric acid.

bamate of gabaculine had no effect on the activity of the enzyme; and (3) the rate of inactivation of the enzyme by gabaculine was unaffected by 50 mM 2-mercaptoethanol at pH 8.6. 2-Mercaptoethanol would have reacted with any alkylating agent free in solution. Experiments that unequivocally demonstrate the requirement of enzymatic turnover of gabaculine prior to the inactivation step are reported later in this section. For the moment, until this proof is offered, let us simply take the requirement for granted.

Assuming, accordingly, that the scheme

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

is representative of the inhibition process with gabaculine, then by plotting $t_{1/2}$ vs. $1/I$, values of K_I and k_{cat} can be calculated assuming $k_{inh} \gg k_{cat}$, (I) $\gg$ (E), and $d(EI)/dt = 0$. This scheme is essentially the one used by Kitz and Wilson (1964) in studying the kinetics of inhibition of acetylcholinesterase by alkylating agents (Jung and Metcalf, 1975). The inactivation data plotted in this fashion yields a $K_I = 2.86 \times 10^{-6}$ M and a $k_{cat} = 1.15 \times 10^{-2}$ s^{-1} at 25 °C for the gabaculine induced inactivation of the enzyme. It is noteworthy that the K_I for gabaculine is approximately three orders of magnitude smaller than the K_M for γ-aminobutyric acid. This could be interpreted to mean that the rigid conformation of gabaculine mimics the active conformer of γ-aminobutyric acid at the active site.

Effect of Substrates on the Rate of Inhibition. If catalytic turnover of gabaculine precedes the inhibition step, then only the pyridoxal form of the enzyme should be susceptible to irreversible inhibition. Therefore amino acid substrates which drive the holoenzyme from pyridoxal form to the pyridoxamine form should prevent inactivation from occurring. Conversely, the addition of an α-keto acid substrate should drive the equilibrium back to the pyridoxal form and hence overcome the protective effect of the amino acid substrate. These predictions have been borne out. In Figure 2 the protective effect of preincubation with γ-aminobutyric acid is shown. Even at concentrations well below its K_M, almost complete protection was observed. The nonsubstrate γ-aminobutyric acid analogue, *N*-methyl-γ-aminobutyric acid, does not afford protection against inactivation. When α-ketoglutarate was introduced to the enzyme preincubated with γ-aminobutyric acid and gabaculine, immediate pseudo-first-order irreversible inhibition of the enzyme ensued as the protective effect of γ-aminobutyric acid was abolished (Figure 3). α-Ketoglutarate by itself had only a small effect on the rate of inactivation by gabaculine (Figure 3). Similar experiments were conducted with D- and L-glutamic acid. L-Glutamic acid protected against inactivation, whereas D-glutamic acid did not. In addition the protective effect afforded by L-glutamic acid was abolished by the addition of α-ketoglutaric acid. α-Keto acids that are not substrates for the enzyme, such as α-ketobutyrate, did not abolish the protective effect of either L-glutamic acid or γ-aminobutyric acid.

pH vs. Rate Studies for the Inactivation Process. If the catalytic turnover of gabaculine is rate limiting in the inactivation process, then the pH vs. rate profile for the inactivation process should be similar to that of normal substrate turnover. The pH optimum for inactivation is approximately 8, whereas that for substrate turnover is 8.6. In both cases, the rates drop off sharply on both sides of the optima (Scott and Jakoby, 1959; Jakoby and Scott, 1959).

Inactivation of γ-Aminobutyric Acid Transaminase by

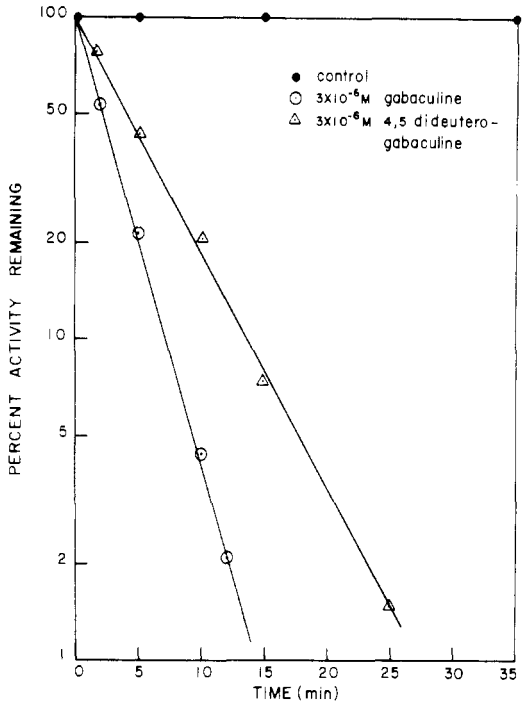
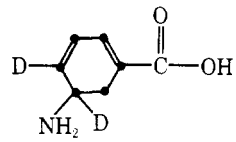


FIGURE 4: Deuterium isotope effect on the rate of inactivation with 4,5-dideuteriogabaculine. Enzyme (0.4 unit) in 0.5 mL of pH 8.6 pyrophosphate buffer was incubated separately with gabaculine at 3×10^{-6} M and with 4,5-dideuteriogabaculine at the same concentrations. The rates of inactivation were determined in the usual manner. ● refers to the control, ⊙ to gabaculine at 3×10^{-6} M, and ▲ to 4,5-dideuteriogabaculine at 3×10^{-6} M. Eighty percent of the 5-position is deuterated in the dideuteriogabaculine. The $k_H/k_D = 2.27$ taking this latter fact into consideration.

4,5-Dideuteriogabaculine. Further evidence for the rate-limiting enzymatic conversion of gabaculine comes from studies on the inhibition of the enzyme by 4,5-dideuteriogabaculine. If C–H bond breakage occurs in the transition state for the conversion, then there should be a deuterium isotope effect on the rate. This in fact occurs and is shown in Figure 4. The measured primary isotope effect was $k_H/k_D = 2.27$. At saturating levels of both gabaculine and 4,5-dideuteriogabaculine, the isotope effect is still manifest. Therefore, the recorded deuterium isotope effect was not caused by a change in binding affinity.



4,5-dideuteriogabaculine

Labeling of the Enzyme with [2-^{3}H]Gabaculine. The enzyme was inhibited with [2-^{3}H]gabaculine (Figure 5). At the indicated times, samples were removed and assayed for remaining activity and count incorporation. As can be seen in Figure 5, inactivation of the enzyme and radioactivity incorporation occurred simultaneously. Radioactivity was not incorporated into the enzyme pretreated with 1 mM γ-aminobutyric acid.

The Enzymatic Formation of m-Carboxyphenylpyridoxamine Phosphate (CPPp). In order to determine whether the enzymatically transformed gabaculine reacted with an active-site residue or the cofactor, labeled enzyme was denatured to see if the radioactivity was releasable. Table I shows that

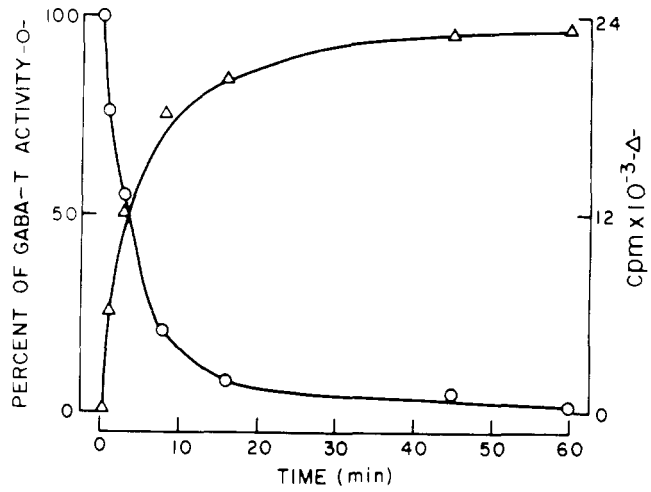


FIGURE 5: Inactivation of γ-aminobutyric acid transaminase with [2-^{3}H]gabaculine. Five units of enzyme (specific activity 2.3 units/mg) in 2 mL of the pyrophosphate buffer was incubated with 3×10^{-6} M [2-^{3}H]gabaculine (specific activity = 17.6 mCi/mmol) at 15 °C. At the indicated times, 5-μL aliquots were removed and the remaining activity was determined. At the same time, 200-μL aliquots were removed and immediately added to 1 mL of 1 mM cold gabaculine in the buffer. The counts incorporated into the enzyme was determined by the Amicon filtration method. In the figure, O refers to the inhibition rate and Δ to radioactivity incorporation. Enzyme pretreated with 1 mM γ-aminobutyric acid was not detectibly inhibited by 3×10^{-6} M gabaculine and after 0.5 h incorporated 100 cpm.

TABLE I: Denaturation of Labeled GABA-Transaminase.[a]

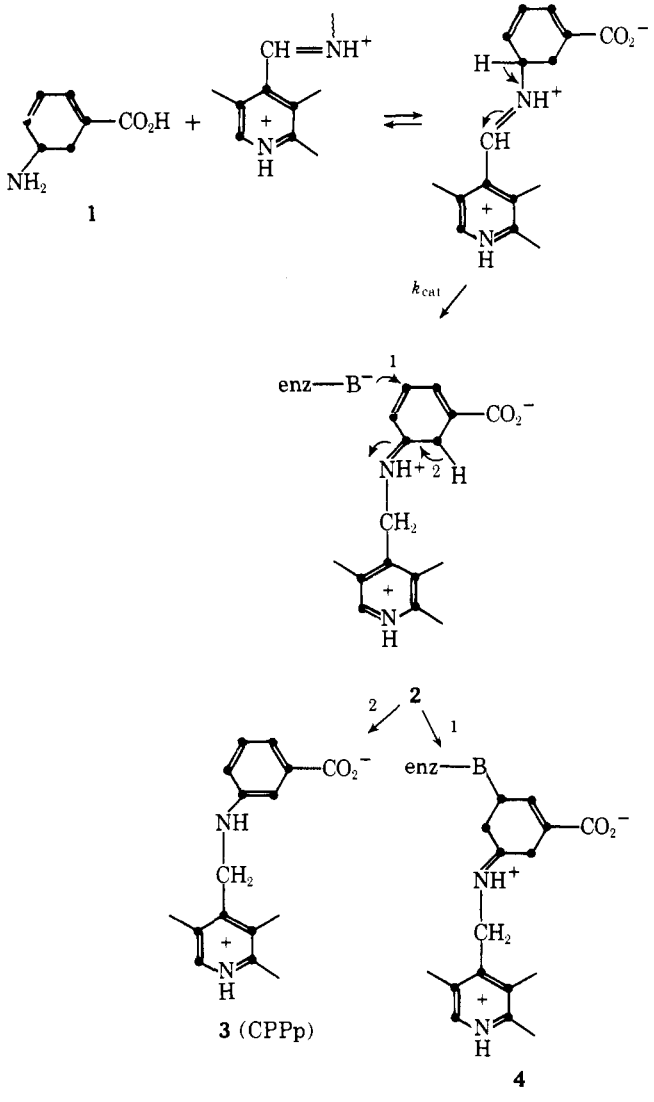
Treatment	cpm (filter)	cpm (filtrate)
Control	4550	200
$NaDodSO_4$ denaturation	425	4287
Heat denaturation	328	4346

[a] Five units of enzyme was labeled with [2-^{3}H]gabaculine as in Figure 6. One unit was added to each of three test tubes containing 1 mL of distilled water (control), 1 mL of 1% $NaDodSO_4$ (sodium dodecyl sulfate) and 40 mM mercaptoethanol ($NaDodSO_4$ denaturation), and 1 mL of distilled water (heat denaturation). The first tube was filtered through the Amicon filtration device and washed several times with water, and radioactivity bound to the filter and appearing in the filtrate was determined. The tube containing $NaDodSO_4$ was heated at 100 °C for 5 min then at 40 °C for 1 h. The second tube containing labeled enzyme in distilled water was heated at 100 °C for 15 min. These latter samples were filtered and the counts bound to the filter and found in the filtrate were determined. The results are given in Table I above.

denaturation of the enzyme led to the clean release of enzyme bound radioactivity. Therefore, a reaction must have ensued between the enzyme activated gabaculine and the pyridoxal phosphate. The reaction product was shown to be CPPp by chromatographic methods. In Figures 6A and 6B the thin-layer chromatographic and paper electrophoretic behavior of the adduct is shown. The compound travelled identically with the carrier CPPp. CPPp could be dephosphorylated by the addition of alkaline phosphate. When the labeled adduct was treated with alkaline phosphatase and then subjected to thin-layer chromatography and paper electrophoresis, it behaved identically with independently synthesized *m*-carboxyphenylpyridoxamine (CPP) (Figures 6C and 6D). These experiments serve to establish the identity of the enzyme catalyzed reaction product of gabaculine and pyridoxal phosphate as CPPp (3).

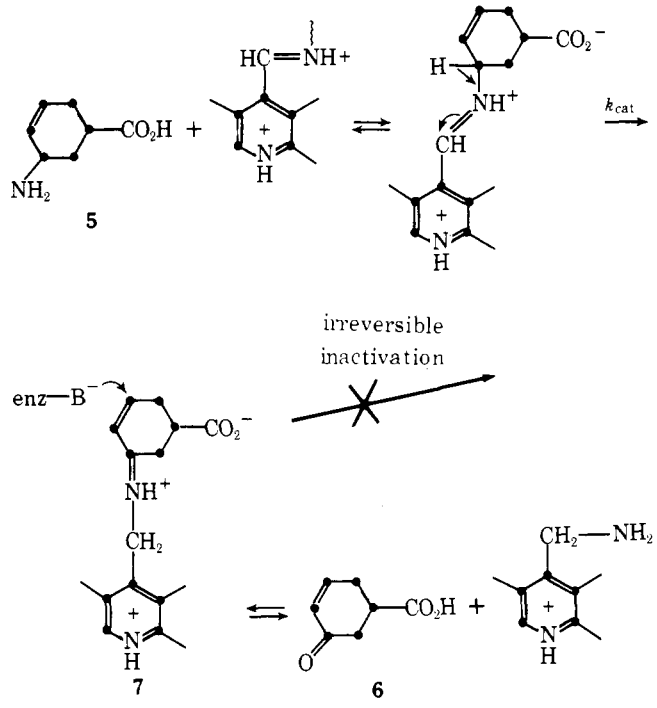
Discussion

The evidence presented here demonstrates that gabaculine is a substrate for bacterial γ-aminobutyric acid transaminase. The rate-limiting step in this conversion is the cleavage of the γ-C–H bond leading to the formation of the chemically reactive intermediate **2.** This intermediate is a Michael acceptor



and is, in principle, capable of alkylating an active-site residue (route 1) instead of undergoing the observed aromatization process (route 2) to generate CPPp. Although the former mode of inhibition is the usual one (cf., e.g., the irreversible inhibition of pyridoxal phosphate linked transaminases by β,γ unsaturated amino acids), it is in the case reported here, evidently not competitive with the aromatization process (Rando et al., 1976).

The aromatization mechanism was directly demonstrated by showing that CPPp was formed enzymatically. Less direct experiments are also consistent with this mechanism. For example, neither 1,2-dihydrogabaculine (*cis* and *trans* mixture) **5** nor 3-keto-4-cyclohexene carboxylic acid **6** are irreversible inhibitors of the enzyme (unpublished experiments). The transaminated intermediate **7** is more chemically reactive than **2,** not having a conjugated double bond, but it, of course, cannot aromatize. Furthermore, simply heating gabaculine and pyridoxal phosphate together results in the nearly quantitative formation of CPPp, **3** (Rando and Bangerter, 1977a). The rate-limiting step on this conversion is the abstraction of



the γ-C–H bond, and the activation energy (E_A) for this process is 24.8 kcal/mol (Rando and Bangerter, 1977a). The activation energy for the enzymatic formation is 10.4 kcal/mol (unpublished experiments). The relatively high E_A for the chemical reaction is the reason why gabaculine is not a general pyridoxal phosphate antagonist. Temperatures of approximately 100 °C and high reactant concentrations are required if the reaction is to proceed at a convenient rate. We have shown that gabaculine is inert as an inhibitor of glutamate decarboxylase, ornithine decarboxylase, aspartate aminotransferase, and alanine aminotransferase when assayed in the millimolar range.

The apparent irreversible inhibition observed when GABA-transaminase is treated with gabaculine results from the tight binding of CPPp generated at the active site. The strength of this binding is not surprising since CPPp is essentially a molecule containing a substrate covalently linked to the cofactor. It is known that pyridoxal phosphate itself is bound very tightly to bacterial γ-aminobutyric acid transaminase (Scott and Jakoby, 1959; Jakoby and Scott, 1959; Baxter and Roberts, 1958). This novel mode of inhibition discussed above should be generally applicable to pyridoxal phosphate linked enzymes. Since these inhibitors do not react with active-site amino acids, they offer the advantage that they can be rationally designed for enzymes whose active-site sequence is undermined.

Although the physiological role(s) of γ-aminobutyric acid transaminase in bacteria has not been clearly defined, its function in mammals has been. γ-Aminobutyric acid is a major inhibitory neurotransmitter found in the central nervous system. The transaminase serves to terminate the action of this neurotransmitter by converting it to succinic acid semialdehyde, a metabolite which is further oxidized to succinic acid by succinic acid semialdehyde dehydrogenase (Wu, 1976).

We have already shown that gabaculine irreversibly inhibits mouse brain γ-aminobutyric acid transaminase (Rando and Bangerter, 1976). It is an exceedingly potent irreversible inhibitor of this enzyme both in vitro and in vivo. When administered intraperitoneally, the transaminase is rapidly inhibited, and the brain levels of the inhibitory neurotransmitter γ-aminobutyric acid rise by 15–20-fold (Rando and Bangerter,

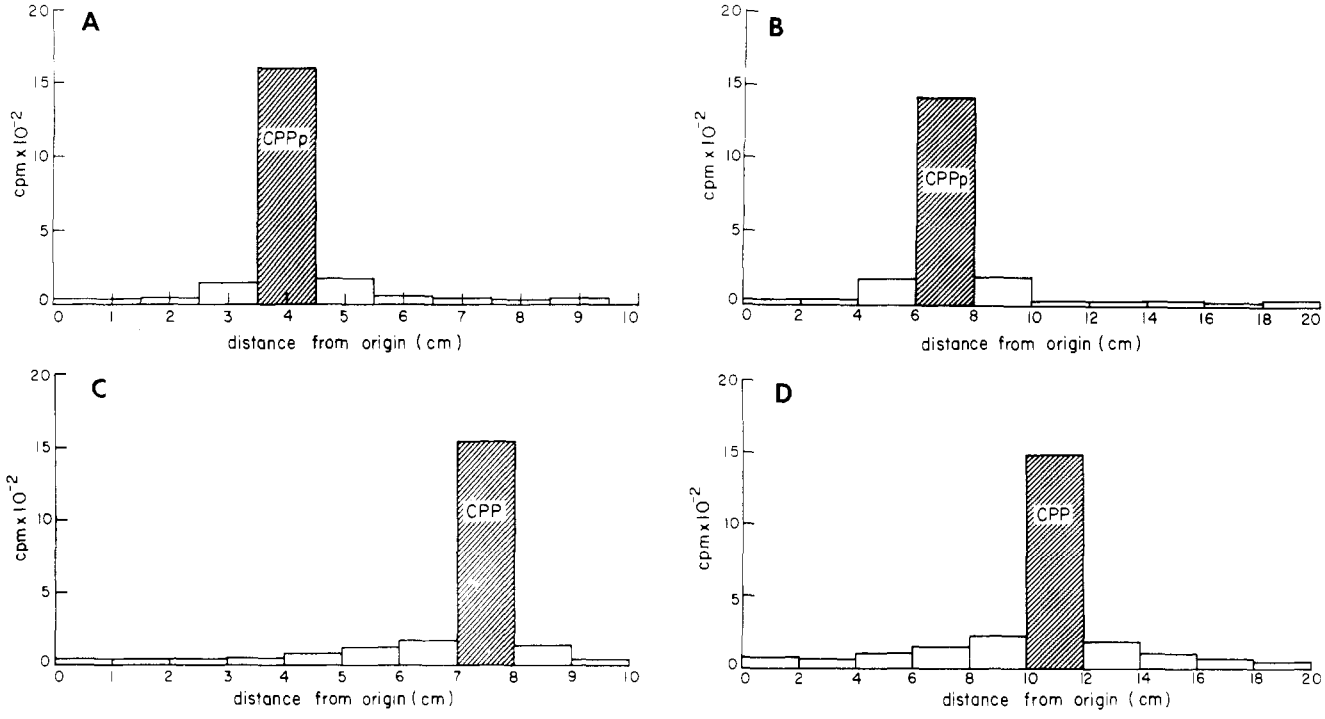


FIGURE 6: The enzymatic formation of CPPp. Five units of purified γ-aminobutyric acid transaminase (specific activity 2.3 units/mg) in 2 mL of the pyrophosphate buffer was completely inhibited by incubating it with 2×10^{-6} M [2-^{3}H]gabaculine (specific activity 17.6 mCi/mmol). The inhibited sample was exhaustively dialyzed against distilled water. This enzyme retained 22 580 cpm. The enzyme was denatured, as in Table I, and filtered through the ultrafiltration device. The filtrate contained 22 155 cpm. This solution was lyophilized to a volume of 1 mL. Five milligrams of cold CPPp was added and aliquots of this solution were spotted on silica gel thin-layer plates and on paper strips. Elution of the thin-layer plates with BuOH-HOAc-H_2O (2:2:1) led to the histogram labeled A. Electrophoresis of the paper strips at pH 1.8 (formic-acetic acid) at 400 V for 1.5 h led to the histogram labeled B. The remaining radioactive solution was adjusted to pH 9 with bicarbonate. Six units of alkaline phosphatase (specific activity 1140 units/mg) was added and the solution was incubated for 30 min at 25 °C. Five milligrams of CPP was added and aliquots of the solution were spotted on thin-layer plates and paper. The thin-layer chromatographic and paper electrophoretic separations were run as before. Histogram labeled C gives the results of the thin-layer separation and the histogram labeled D gives the results of the paper electrophoretic separation. In all cases between 2100 and 2400 cpm was spotted. The inked in histograms refer to where either CPPp or CPP appears.

1977b). Thus gabaculine promises to be a powerful pharmacological tool in the study of gabanergic neurotransmission.

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